MOLECULAR CHARACTERISATION OF *TRYPANOSOMA EVANSI* STOCKS FROM INDONESIA

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DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institute of tertiary education. Wherever contributions from colleagues have been included this has been fully acknowledged and a list of references is given.

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ABSTRACT OF THESIS

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The work described in this thesis used the techniques of karyotype analysis, random amplified polymorphic DNA analysis (RAPD), Riboprinting and simple sequence repeat polymerase chain reaction (SSR-PCR) to examine genetic variation in stocks of Indonesian *Trypanosoma evansi*. The 80 *T. evansi* stocks investigated were isolated from natural infections in cattle, buffaloes and horses in 10 widely separated areas, including Aceh, North Sumatra, Lampung, West, central and East Java, Madura, South Kalimantan, North and South Sulawesi.

Transverse alternating field electrophoresis (TAFE) was used to determine the polymorphisms present in the karyotype patterns of the 80 *T. evansi* stocks. Electrophoretic conditions were developed to give good resolution of chromosome bands in the size range 50 kb to 5.7 Mb. The total number of chromosome bands in the *T.evansi* genome varied from 17-27 depending on the stock.

A high degree of polymorphism was detected in the karyotype patterns of *T. evansi* stocks from Indonesia by TAFE. In the 80 stocks studied, 46 different karyotype patterns were detected. Cluster analysis with a grouping level of 10% fitted the patterns into seven main karyotype groups which correlated with the locality from which the stocks had been isolated. The stability of the karyotype patterns within stocks was not related to differences between the surface variant antigenic types (VAT) in that relapse populations derived from a single infection with a cloned stock of *T. evansi* showed the identical karyotype pattern to the original infecting stock. In relapse populations collected from an uncloned *T.evansi* stock different karyotype patterns were found indicating that more than one population was present in the original isolate. Attempts to isolate VAT specific chromosomal probes were not successful.

The results from karyotype analysis of *T. evansi* were shown to be sufficiently stable with regard to the number, sizes, and intensities of the chromosomal bands seen 1) among different cryopreserved preparations of the same strain or clone; 2) after passage through laboratory animals; 3) among different agarose embedded DNA preparation of the same stock or clone; 4) among relapse populations collected from a single infection with a cloned stock; 5) between stocks sensitive and resistant to trypanocidal drugs such as suramin and cymelarsan.

Karyotype analysis of *T. evansi* stocks isolated from buffaloes transported from central Java to North Sumatra indicated that *T. evansi* infection had been transferred from the local to the transported buffaloes, not vice-versa, during a period of 13 months after transportation. A high degree of similarity in karyotypes was observed in *T. evansi* stocks isolated from a group of Bali cattle kept together in a feedlot in Lampung indicating that a single *T. evansi* stock of a particular pattern was responsible for the infection in this outbreak. Stocks collected from West and central Java during a six-year period from 1988-1994 showed a high degree of karyotype patterns stability.

The potential polymorphisms with regard to chromosomal locations of several genetic markers including Phospholipase C, Cysteine Proteinase, *T. brucei* ribosomal RNA coding region, *T. brucei* tubulin, Aldolase and Glucose 6-phosphate isomerase were determined in *T. evansi*, *T. brucei* and *T. congolense*. The hybridisation patterns correlated with the karyotype pattern shown by each stock of *T.evansi*. The hybridisation patterns shown by *T. evansi* stocks were different to those of *T. brucei* and *T. congolense*.

Random amplified polymorphic DNA (RAPD) analysis of the 80 *T. evansi* stocks studied yielded only four RAPD patterns. *Trypanosoma evansi* stocks isolated from a group of buffaloes transported from central Java to North Sumatra showed RAPD patterns that indicated an introduction of *T. evansi* infection in the central Java buffaloes by North Sumatra stocks during a 13 months period after the transportation. Polymorphism in the RAPD patterns was also detected in *T. evansi* stocks isolated from a group of Bali cattle kept in a feedlot in Lampung, a particular RAPD pattern was detected in most of the stocks collected. *Trypanosoma evansi* stocks isolated from relapse populations, however, showed identical RAPD pattern.

This study showed that RAPD analysis is useful for characterising *T. evansi* stocks and could be used to analyse a large number of samples collected from widely distributed areas. The sample preparation for RAPD analysis can be simple, the DNA can be extracted directly from cryopreserved stabilates and the results obtained in the same day.

The riboprinting applied in the study did not detect variations among *T. evansi* stocks. Variation in riboprint banding patterns was shown between the *T. evansi*, *T. brucei* and *T. congolense* stocks. The simple sequence repeat PCR (SSR-PCR) assay showed the least specificity. The analysis generated complex banding patterns and polymorphisms in the banding pattern were not easily detected.

A number of molecular techniques were used to compare morphologically identical stocks of *T. evansi* in Indonesia. The results demonstrated greater heterogeneity in *T. evansi* than previously reported by other workers with stocks of *T.evansi* from other areas of the world. This study showed that of the methods used , karyotype analysis by TAFE detected stock variations at a finer level than RAPD analysis, riboprinting and the SSR-PCR assay. The RAPD analysis is, however, preferable for epidemiological studies due to the technical simplicity of performing the assay and the ease of sample preparation.

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LIST OF ABBREVIATIONS

AGT	=	Agglutination Test for Trypanosomes
ALD	=	Aldolase
BAKIT	=	Balai Penelitian Penyakit Hewan
bp	=	basepairs
CHEF	=	Contour-clamped Homogeneous Electric Field
CP	=	Cysteine Proteinase
DEAE52	=	diethyl aminoethyl 52
DNA	=	Deoxyribonucleic acid
dNTPs	=	deoxyribonucleic acid triphosphates
EDTA	=	Ethylene diamine tetraacetic acid
ELISA	=	Enzyme-linked immunosorbent assay
ES	=	Expression sites
FIGE	=	Field inversion gel electrophoresis
gRNA	=	guide RNA
kb	=	kilobasepairs
kDNA	=	kinetoplast DNA
LMP	=	Low melting point
Mb	=	Megabasepairs
MHCT	=	Microhaematocrit centrifugation technique
OFAGE	=	Orthogonal field alternation gel electrophoresis
PACE	=	Programmable autonomously controlled electrodes
PCR	=	Polymerase Chain Reaction
PFGE	=	Pulsed field gel electrophoresis
PGI	=	Glucose-6-phosphate isomerase
PHOGE	=	Pulsed homogeneous orthogonal field gel electrophoresis
PLC	=	Phospholipase C
PSG	=	Phosphate Saline-Glucose
RAPD	=	Random Amplified Polymorphic DNA
RFLP	=	Restriction fragment length polymorphism
RGE	=	Rotating gel electrophoresis
RNA	=	Ribonucleic acid
rRNA	=	ribosomal RNA
SSR-PCR	=	Simple Sequence Repeat-Polymerase Chain Reaction
ST/RIDE	=	Simultaneous tangential/rectangular inversion decussate electrophoresis
T_A	=	Annealing temperature
TAFE	=	Transverse Alternating Field Electrophoresis
TREU	=	Trypanosoma Research Edinburgh University
UV	=	ultraviolet
VAT	=	Variant antigenic type
VSG	=	Variant surface glycoprotein

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CHAPTER ONE

INTRODUCTION AND OBJECTIVES

In Indonesia, surra first appeared in horses and buffaloes in Java in 1886 (Penning, 1903). It appeared in Roti (near Timor) in 1894 and in Sumatra in 1901 (Schat, 1902). Despite the measures taken to control the disease, surra was enzootic throughout low-lying areas of Java by 1917 (Doeve, 1917). Between 1920 – 1927 some 25,000 cases of surra were recorded and 25% of them in horses and all fatal (Bakker, 1930). There is little recent information about the incidence and economic importance of *T. evansi* which means there is a need to obtain data on the epidemiology of surra in endemic regions. In areas where surra was previously a problem the incidence now appears to be low. This may be due to control measures or several changes, such as agricultural practices and transport means reducing the number of target animals. Also, because the disease is chronic in buffaloes and cattle, and since parasitological diagnosis is inefficient and modern serological tests have not yet been applied, the incidence of surra is, therefore, probably much greater than it appears.

In Indonesia, surra is still a major problem and although infection rates, and mortality in cattle and buffalo are low, severe epidemics still occur, mainly in Java (Rukmana, 1979). In Java an outbreak of surra in 1968 – 1969 killed >3,000 animals, involved screening of 170,000 and treatment of 8,500 (Adiwinata and Dachlan, 1969; Soetrisno, 1970). Surra in horses in Indonesia is usually rapid and fatal. Cattle and buffalo can suffer acute or chronic infections with the possibility of subclinical infections being reservoirs of infection.

The widespread geographical distribution, the range of susceptible hosts, the range of clinical symptoms and further pathogenicity all suggest that *T. evansi* may be represented by a range of genotypes. The evidence for a wide range of diversity is not strong. However, there is little diversity in *T. evansi* stocks from Sudan, as measured by isoenzyme analysis; karyotype analysis revealed only limited differences in *T. evansi* stocks from Kenya and China; antigen diversity in *T. evansi* showed that the Sudanese stocks contain 3 serodemes and Indonesia isolates consisted of 1 major and 5 minor serodemes.

One of the primary problems in parasite epidemiology is the correlation of disease with its causative organism. In the case of the morphologically identical trypanosomes of *T. evansi*, morphologically identical parasites can have different pathogenicities for different hosts.

These differences might be explained at the genetic level. By analysing trypanosome DNA using modern molecular biology techniques the following questions can be addressed:

1. Can the species or strain causing a particular clinical status be identified or differed? The identification of species or strain is very important in the case of morphologically similar parasite species that infect and can co-exist in a range of hosts.

2. What vectors or intermediate hosts can carry the disease?

3. What strain/variants are associated with disease status, or outbreaks or epidemics?

Answers to these questions will help to define the disease carrying organism and its distribution.

There are a large number of genotypes in hosts and vectors, which can determine disease incidence, transmission and severity of symptoms. All of these need to be analysed in order to understand the epidemiology of parasites.

Much effort has been put into identifying parasites, and the variation within and between groups of parasites, in order to try to provide the answer to epidemiological questions. Such techniques can be used for an absolute identification between the member parasite species or can be used to examine fine differences between members of the same species or individuals within a group. Comparisons between species and strains can be made using a number of techniques that study variation at the DNA level, such as, hybridisation; DNA polymorphism (RLFP); DNA sequencing; analysis of molecular karyotype, analysis of nuclear and extra nuclear DNA; and the polymerase chain reaction.

The genetic diversity found in any population of any species can originate from gene mutation, recombination and gene flow from other populations. These processes especially recombination, are important in generating diversity in parasite protozoa. Recombination in *T. brucei* has been demonstrated by Gibson, Marshall and Godfrey (1980), Tait (1980) and Jenni, Schweizer, Betschart *et al.* (1986) but no evidence of such genetic recombination has been reported in *T. evansi.* This does not rule out extensive diversity in *T. evansi* because Mayr (1963) noted that an organism that reproduces by rapid asexual division, even at a low mutation rate, can provide enough variability for the organisms to survive in a slowly changing environment. However, for the majority of micro-organisms a phase of genetic recombination occurs which allows a large increase in the number of different genotypes produced.

The trypanosome genome is organised in a large number of individual DNA elements termed chromosomes that can be revealed by pulsed field gel electrophoresis (PFGE). The chromosomes of trypanosome are organised as an undetermined number of large chromosomes (>1000 kb), a few intermediate sized chromosomes (200 – 700 kb) and

numerous mini chromosomes (50 – 150 kb). The precise numbers in each category is unknown and varies from one parasite to another. The organism is either diploid (Gibson, Osinga, Michels, *et al.*, 1985), triploid (Gibson, Garside and Bailey, 1992; Gibson and Bailey, 1994) or sub-tetraploid (Paindavoine, Zampetti-Bosseler, Pays, *et al.*, 1986a; Wells, Prospero, Jenni, *et al.*, 1987; Gibson *et al.*, 1992; Gibson and Bailey, 1994) and some genes are present in the genome in only single copies.

The most studied characteristics of trypanosomes are phenotypic characters such as variations in infectivity to vertebrate hosts, morphology, life cycle details, antigens and enzymes. Studies at the molecular level however can be used to reveal differences in DNA that can be related to epidemiological feature of the parasite.

The present study was carried out to try to obtain a better understanding of genetic diversity in *T. evansi* in relation to epidemiological features. By using a range of molecular-based technologies such as karyotyping, random amplified polymorphic DNA (RAPD) analysis, riboprinting and the simple sequence repeat polymerase chain reaction (SSR.PCR) it was hoped to identify marker systems for epidemiologically significant features of *T. evansi* that could be used in epidemiological studies on the parasite. It was hoped that through the use of a range of technologies suitable marker systems would be identified that could ~~be enabling~~ differentiate ~~of~~ stocks with ~~in~~ regard to their susceptibility to trypanocidal drugs, their origin in particular hosts which they were isolated, their geographical location, pathogenicity and changes in the variant antigenic types.

CHAPTER TWO

LITERATURE REVIEW

2.1. INTRODUCTION

Trypanosomiasis is a disease which affects humans and their livestock in many countries throughout the Old and New Worlds. Trypanosomes of economic importance can be divided into two groups according to their mode of transmission. Cyclically transmitted trypanosomes undergo a developmental cycle in the tsetse-fly vector; mechanically transmitted trypanosomes do not have any part of their life cycle in the fly vector. Both groups are physically transmitted from one host to another by the insect vector. The following species of trypanosomes are of veterinary importance. *Trypanosoma equiperdum* is a species that affects equids and is transmitted by coitus. The cyclically transmitted trypanosomes, *Trypanosoma brucei*, *T. congolense* and *T. vivax* are transmitted by tsetse flies of the genus *Glossina* and restricted to Africa except for certain isolates of *T.vivax* which are transmitted mechanically by biting flies and occur in South America. *Trypanosoma evansi* is mechanically transmitted and has the widest distribution of all the trypanosome species occurring in central and southern America, through the Middle East, eastern Europe, central Asia, India, Thailand, Indonesia, the Philippines, Vietnam and China.

2.2. *TRYPANOSOMA EVANSI* IN INDONESIA

According to the "*T. brucei* origin of *T. evansi*" theory (Leese, 1927; Godfrey and Killick-Kendrick, 1962), caravans of camels temporarily taken from North Africa and entering the tsetse area in the South of the Saharan desert were exposed to *T. brucei* infection. When these camels left the tsetse belt and returned to the North the trypanosomes became adapted to a mechanical means of transmission and spread the infection among animals in the absence of tsetse flies. Having acquired chronic infection, camels spread *T. evansi* as they travelled in caravans across sub-Saharan Africa, the Arabian Peninsula, Asia Minor and India (Curasson, 1943). The spread of surra can therefore be attributed to the camel in its capacity as a beast of burden for transport in trading and military missions in Africa and the Middle East. Other livestock such as horses, buffaloes, and cattle acquired surra when they

were exposed to infected camels. Further spread of surra across into South America and islands including Mauritius, the Philippines and Indonesia can be traced to the importation of infected livestock from endemic areas (Dieleman, 1983). Surra was first discovered in Indonesia by Penning in 1897 (Penning, 1903) who wrote that a disease imitating surra in India was recognised in Indonesia at the beginning of 1897. Penning stated that surra occurred all over Java, Sumatra and Sulawesi. The death of some horses on the islands of Sawo and Roti (between Sumba and Timor) over a period of several years might also be due to *T. evansi* infection (Penning, 1903).

Surra is considered as one of the most important disease in livestock in Indonesia (Rodenwaldt and Douwes, 1921; Bakker, 1930; van Zwieten, 1932; Adiwinata and Dachlan, 1969). Widespread endemicity of *T. evansi* has been reported on the main islands of the Indonesian archipelago (Adiwinata and Dachlan, 1969; Payne, Sukanto, Djauhari *et al.*, 1991a). Serological evidence of *T. evansi* infection in cattle, buffaloes and horses in Indonesia is shown in **Table 2.1**. The prevalence of serologically positive reactors was higher in buffaloes than in cattle in areas where cattle and buffaloes were sampled together. Similarly, the prevalence of parasitologically positive reactors (**Table 2.1**) was higher in buffaloes than in cattle, however, evidence of *T. evansi* infection in horses was not detected (Payne *et al.*, 1991a). The buffaloes are used for draught purposes in the irrigated rice fields an environment which probably supports a high population of the fly vectors. Infection rates may also be related to the feeding preferences of the flies (Payne *et al.*, 1991a). *Trypanosoma evansi* infection rates in horses in Indonesia are usually low (Payne *et al.*, 1991a) since the horses are frequently protected chemotherapeutically as soon as a surra outbreak is suspected.

Among the wild animals in Indonesia, the grey Javan monkey (*Macaca irus mordax*), Chital deer (*Axis axis*), Kangean deer (*Rusa timorensis* subspecies), the Indonesian black-neck hare (*Lepus nigricollis*) and the woodcat (*Prionailurus bengalensis javanensis*) can acquire infection with *T. evansi* and develop chronic infections (Kraneveld and Mansjoer, 1952). The acquisition of *T. evansi* infection raises the possibility that wild animals may act as reservoir hosts for surra. Extensive farming systems are practised by groups of local farmers in Irian Jaya, Sulawesi, Kalimantan and Sumatra where animals are grazed in areas near forest. Since these wild animals come into contact with the grazing livestock, they may act as reservoir hosts and provide a source of *T. evansi* infection for domesticated animals.

Table 2.1. ***Trypanosoma evansi*** **infection rate in livestock in different areas of Indonesia determined by parasitological (MHCT, microhaematocrit centrifugation technique) and serological (ELISA, enzyme-linked immunosorbent assay) examinations.**

Area	Cattle			Buffalo			Horses		
	Total sampled	MHCT (% Infected)	ELISA (% positive)	Total sampled	MHCT (% Infected)	ELISA (% positive)	Total sampled	MHCT (% Infected)	ELISA (% positive)
Aceh[1)]	287	0.3	46.7	0	-	-	26	0	3.8
East Sumba[1)]	184	0	17.9	112	0	28.6	41	0	0
Lampung[1)]	271	5.2	64.2	31	25.8	87.1	0	-	-
South Sulawesi[1)]	50	2.0	32.0	0	-	-	31	0	3.2
North Sulawesi[1)]	81	1.2	37	0	-	-	142	0	2.8
West Timor[1)]	291	ND	38.1	0	-	-	0	-	-
South Kalimantan[1)]	358	1.4	42.5	30	0	10	0	-	-
Central Java[1)]	0	-	-	103	5.8	70	107	0	1.7
Yogyakarta[1)]	0	0		74	5.4	67	0	0	
Madura[2)]	130	13.1	30	147	49.6	47	0	-	-
East Java[3)]	63	1.6	3	154	0.6	32	0	-	-
Overall	1741	2.3		639	14.4	36.6	347	0	2.3

1) Payne *et al.*, 1991a.

2) Sukanto *et al.*, 1988.

3) Sukanto, 1992.

Naïve imported animals suffered more severe infection with *T. evansi* than local animals and high mortality rates were reported in the imported animals (Waltner-Toews, Payne, Djauhari *et al.*, 1988; Doeve, 1917). It is the Government of Indonesia's policy to import cattle and buffaloes into Indonesia to improve the productivity of the local livestock and to increase the number of smallholder farmers raising buffaloes for draught purposes. During the first year of their arrival in Aceh after importation from New Zealand, Sahiwal crosses suffered high mortality rates due to haemoparasite infections (Ward, 1986; Payne, Ward, Usman *et al.*, 1988). Buffaloes imported from northern Australia suffered a similar fate (Waltner-Toews, 1987) which was later shown to be caused by *T. evansi* infections (Waltner-Toews *et al.*, 1988).

Surra outbreaks have occurred regularly in Indonesia (Douwes, 1923; 1924; Bakker, 1930; van Zwieten, 1932; Adiwinata and Dachlan 1969) particularly in Sumatra (van Zwieten, 1932). Veterinary reports published since 1975 record many surra cases each year in Java. Diagnosis of surra, however, is still carried out by examination of blood smears, which has a low sensitivity and most likely underestimates the true number of animals infected with *T. evansi* in Indonesia. Surra outbreaks occurred among cattle and buffaloes in Central Java between 1968-1969 (Adiwinata and Dachlan, 1969); in Madura and East Java during 1988-1989 (Sukanto, Payne and Graydon, 1988; Payne, Sukanto, Graydon *et al.*, 1990; Yearly reports of the Dinas Peternakan Propinsi Jawa Timur 1988/1989); and in South Kalimantan during 1992 (Djauhari, personal communications).

A number of studies, which have included the identification of arthropod vectors that may play a role in the transmission of *T. evansi*, have been carried out in Indonesia since 1925. Different arthropod species implicated in the transmission of *T. evansi* in Indonesia included *Haematopota* (Nieschulz, 1927a; Nieschulz and Ponto, 1927a), *Chrysops* (Nieschulz and Ponto, 1927b), *Stomoxys, Lyperosia, Musca, Stegomyia* (Nieschulz, 1927b), *Tabanus* (Nieschulz, 1925; 1927a; c; d; e; 1928a; b; Nieschulz and Ponto, 1927a; c) and *Anopheles* (Nieschulz, 1928c). Although *Tabanids* were suggested as the most likely vectors for *T. evansi* by early transmission studies (Niezchulz, 1927a; 1930), later studies carried out from 1992-1996 indicated that *Stomoxys* and *Haematobia* were the most prevalent flies caught in *T. evansi* endemic areas in Java. The tabanids are normally found in wet areas in rice fields and forest (Nieschulz, 1927f), and the animals are infested by the flies when the animals are washed. Flies are subsequently brought back to the animal shelters by the buffaloes.

2.3. FACTORS CAUSING DIVERSITY IN *T. EVANSI*

The importance of characterising trypanosomes is not just to assign them to their correct taxonomic group but to identify the particular species and strains involved in an infection in

order to decide on appropriate control measures (Godfrey, 1978). In morphologically homogeneous species, such as *T. evansi*, differences among stocks are often found in pathogenicity, responses to trypanocidal drugs and host preferences.

Difficulties in distinguishing trypanosomes of different species, sub species or strains arise because of the similarity of their appearance. To achieve this characterisation, various features are used, either singly or more usually in combination to identify trypanosomes. These include morphology, host specificity, pathogenicity, drug resistance, isoenzymes and antigenic diversity and the more modern methods for characterisation such as molecular based techniques.

2.3.1. DIVERSITY IN MORPHOLOGY

Trypanosoma evansi can be easily distinguished from *T. congolense*, *T. vivax* and *T. simiae* by its size and position of the kinetoplast and by the presence of a conspicuous undulating membrane and free flagellum. The morphological appearance of *T. evansi* is, however, indistinguishable from the other members of the genus *Trypanozoon; T. brucei* and *T. equiperdum* (Hoare, 1972). *Trypanosoma brucei* is pleomorphic and has been shown to contain two morphologically distinct trypanosome populations, namely short stumpy and long slender forms (Hoare, 1972). *T. evansi* is principally monomorphic, however, some stage of pleomorphism including the presence of typical and posteronuclear stumpy forms as found in *T. brucei* is occasionally observed. An association has been found between the length of trypanosomes and their pathogenicity to different hosts in tsetse-transmitted trypanosomes but no such association has been observed for the *T. evansi* stocks studied.

Phenotypically, *T. evansi* varies in length, and measures from 15 to 34 μm (Hoare, 1972). *Trypanosoma evansi* stocks from Sudan, Colombia and Kenya are monomorphic and measured from 19 to 33 μm with a mean length of 25.5 ± 2.8 μm. The *T. evansi* stock from Colombia had the shortest length (19.4 ± 0.8 μm) (Mutugi, 1993).

Variation in the length of *T. evansi* stocks was also observed in the Indonesian stocks collected. Holz (1964) detected the long type of *T. evansi* in animals with new infection and the short forms in blood samples from animals with chronic infection. Djuhaifah (1966) measured a *T. evansi* Bogor stock from 13.8-16.3 μm for the short types and 17.2-24 μm for the long *T. evansi* forms.

Although there is a wide variation in length among *T. evansi* stocks from widely distributed areas, the size of *T. evansi* measured are still within the range stated by Hoare (1972) and the difference in length is more likely to be due to individual trypanosome variation and not the presence of distinct populations.

2.3.2. DIVERSITY IN HOST PREFERENCE AND VIRULENCE

Trypanosoma evansi can be pathogenic to all domestic animals with disease symptoms that differ in severity according to the strain virulence and host susceptibility. Unless treatment is applied, surra is fatal in horses and dogs and these animals do not survive the infection to become chronically infected reservoir hosts (Rodenwaldt and Douwes, 1921; Douwes, 1923; Bakker, 1930).

The incubation period in horses naturally infected with surra varies from 4 to 13 days (Nieschulz and Kraneveld, 1928). The infection is usually acute with up to 100% mortality rates and death occurring in 4 to 8 weeks in untreated horses (Rodenwaldt and Douwes, 1921; Douwes, 1923; Bakker, 1930). When the central nervous system is affected the death can occur within 3 to 4 weeks (Rodenwaldt and Douwes, 1921; Douwes, 1923; Bakker, 1930). It would therefore appear that horses are inherently susceptible to *T. evansi* infection and that the pathogenicity seen in horses is a feature of the host and not due to diversity of the trypanosome.

Cattle and buffaloes usually undergo chronic *T. evansi* infection with an incubation period, which varies from 7 to 13 days (Rodenwaldt and Douwes, 1921; Douwes, 1923; Bakker, 1930). The mortality rates are generally low and latent infections form reservoirs of the parasite (Rodenwaldt and Douwes, 1921; Adiwinata and Dachlan, 1969) which are possible sources of infection for susceptible animals (Bakker, 1930; Nieschulz, 1927g; 1930). Although the mortality rates are generally low, severe outbreaks of disease can occur with buffaloes experiencing more severe reactions than cattle (Adiwinata and Dachlan, 1969; Lohr, Pholpark, Srikitjakarn, *et al.*, 1985; Sukanto *et al.*, 1988; Payne *et al.*, 1990). The reasons for higher infection rates in buffaloes by *T. evansi* are not known, but may be related to the higher levels of fly challenge in the areas where the buffaloes are kept and worked.

In many cases buffaloes and cattle suffering from *T. evansi* infection can overcome the infection without treatment (Kraneveld and Djaenoedin, 1948). There are, however, cases of animals in good condition, dying without any symptoms where trypanosomes were only revealed at post-mortem investigation (Rodenwaldt and Douwes, 1921; Douwes, 1923; Bakker, 1930). In chronically infected animals, clinical disease may not become apparent as long as the animals receive reasonably good nursing and food. On the contrary, when the animals are poorly fed and under work stress an acute deteriorating infection may appear. The severity of *T. evansi* infection was found to be higher when *T. evansi* infected animals were co-infested with *Fasciola gigantica* and subjected to seasonal malnutrition during dry season (Lohr *et al.*, 1985).

Sheep, goat and pigs appear not to be susceptible to surra although they can become infected, they seldom show any clinical symptoms (Bakker, 1930). Dogs and cats are very

susceptible to surra with mortality rates as high as 100% (Bakker, 1930; Adiwinata, 1957). Without treatment cats may die within 17-53 days (Penning, 1903, Adiwinata, 1957) and dogs within 1 to 3 months (Bakker, 1930).

2.3.3. ANTIGENIC DIVERSITY

Antigenic diversity in trypanosomes is seen as differences between serodeme expression by different parasite populations. Antigenic variation is a property of antigenic differences of populations of single infection. A serodeme is a group of trypanosome populations with the same range of variants. The phenomenon of antigenic variation is based on trypanosomes possessing several hundred VSG gene copies within serodemes which can be expressed during the course of an infection in order to evade the host's immune response. Although the appearances of the VSG sequences are random, and only one VSG is expressed at one time, predominant VATs have been demonstrated within *T. evansi* serodemes that appear early in infection and can be used to characterise stocks.

The parasites have several hundred gene copies of VSG and each copy is expressed at different times therefore the surface coat also changes evading the host's immune response which has developed to the previously expressed copy.

A majority of the VSG genes are located on the intermediate and megabase size chromosomes (Van der Ploeg, Schwartz, Cantor, *et al.*, 1984a) but only those within telomeric expression sites (ES) are expressed (Myler, Allison, Agabian *et al.*, 1984). Many of the mini-chromosomes (50-150 kb size range) carry VSG genes at their ends and may act as reservoirs of VSG genes (Van der Ploeg *et al.*, 1984a).

Since the VSG genes expressed by the parasite are numerous, the number of variant antigenic types (VATs) in a population can be large. The largest number of different VATs observed in a single laboratory infection of *T. equiperdum* was 101 (Capbern, Giroud, Baltz *et al.*, 1977). Different trypanosome stocks, however, do not always possess the same VAT repertoire (Van Meirvenne, Janssens and Magnus, 1975a; Van Meirvenne, Janssens, Magnus, *et al.*, 1975b; Van Meirvenne, Magnus and Vervoort, 1977).

Trypanosome populations that express the same VAT repertoire are referred to as a serodeme. Jones, Cunningham, Taylor *et al.* (1981) showed very limited diversity in the serodemes within stocks of *T.b. gambiense* with one major and several minor serodemes. Studies on *T.b. brucei* and *T.b. gambiense* have suggested that predominant VATs tend to appear regularly during the early infection in different hosts which were infected with the same stock (Gray, 1965; 1972; 1975); these predominant VATs are considered to be characteristics in a particular stock (Gray, 1965).

Relapse populations of a single stock have been used for classification of trypanosome stocks based on their predominant VAT repertoires (Van Meirvenne *et al.*, 1975a, b; 1977). Jones and McKinnel (1985) have applied a similar approach and it was suggested that the VAT composition obtained from the first relapse populations is useful for classification of *T. evansi* stocks into serodemes based on their predominant VAT repertoires. Jones and McKinnel (1984) showed that 10 out of 11 VATs populations, collected from relapse populations of a single *T. evansi* infection, represent predominant VATs of the stock. This suggested that *T. evansi* stocks could be classified according to their serodemes as in *T. brucei* (Gray and Luckins, 1976), which is also useful for epidemiological studies (Jones and McKinnel, 1984). Serodeme analysis is a labour intensive procedure, which depends on the availability of trypanosome stocks representing the full range of VATs. It also requires mice to expand the trypanosome stocks. Although serodeme analysis is a useful procedure for characterising trypanosomes, the technique is not impractical for the comparison of large numbers of stocks.

2.3.4. ISOENZYMES

Isoenzyme analysis has been applied to epidemiological and taxonomic studies to differentiate subspecies and strains of *Trypanozoon* (Tait, Barry, Wink *et al.*, 1985; Stevens and Godfrey, 1992; Stevens, Lanham, Allingham, *et al.*, 1992; Cibulkis, 1992) and *T. vivax* (Fasogbon, Knowles and Gardiner, 1990). Isoenzymes are multiple molecular types of particular enzymes, which have similar properties but differ in their electrophoretic mobilities due to differences in charge of the different molecular structures. Changes in the molecular structures occur at the level of gene transcription. The occurrence of multiple gene loci coding for the enzyme polypeptide chains is the source of structural differences. Mutations in the gene loci accumulated during evolution lead to structural changes in the enzymes and eventually lead to interspecific enzyme differences (Harris and Hopkinson, 1976). Structural and post-translational variations may not change the function of the resulting enzymes but may result in a change in the overall net charge leading to differences in electrophoretic mobilities (Harris and Hopkinson, 1976). A second source of isoenzymes is the occurrence of multiple alleles at a single gene locus, which leads to either homozygous or heterozygous phenotypes. Depending on the number of alleles at a particular locus various combinations of the alleles are possible; the complexity of which result in intraspecific differences.

Isoenzymes and their mobility differences in starch gels have been used to characterise different species in *Entamoeba* (Reeves and Biscoff, 1968), *Plasmodium* (Carter, 1973), *Naegleria* (Musisi, 1978; Melrose, 1983) as well as *Leishmania* (Chance, Schnur, Thomas, *et al.*, 1978) and *Trypanosoma* (Kilgour and Godfrey, 1973; Murray, 1982; Godfrey, Scott, Gibson, *et al.*, 1987).

Isoenzyme analysis has been used for the identification of species, subspecies and strain groups within the subgenus *Trypanozoon* (Stevens *et al.*, 1992) and establishing a numerically-based taxonomy for *Trypanozoon* (Stevens and Godfrey, 1992). It was concluded that *T. evansi* and *T.b. gambiense* stand as well-defined species and subspecies of *T.b. brucei* (Stevens and Godfrey, 1992). Godfrey *et al.* (1987) examined 32 isolates collected from known Gambian trypanosomiasis areas in Sudan, Kenya, Zaire, Nigeria, Ivory Coast, Burkina Faso, Liberia and Senegal using isoenzyme electrophoresis of 11 enzymes. The existence of a homogeneous group was observed in trypanosomiasis throughout tropical Africa based on certain isoenzyme combinations and low initial virulence to rodents which is the classical concept of *T.b. gambiense* (Godfrey *et al.*, 1987). Earlier results showed that there was no enzyme variants specific to *T.b. rhodesiense* and it was concluded that *T.b. rhodesiense* is a set of *T.b. brucei* variants rather than a subspecies (Tait *et al.*, 1985). This result was confirmed at the DNA level, and showed that *T.b. gambiense* was distinguishable from non-gambiense (Paindavoine, Pays, Laurent, *et al.*, 1986b; Paindavoine, Zampetti-Bosseler, Coquelet *et al.*, 1989).

Isoenzyme analysis has been applied extensively to characterise *T. evansi* stocks. Gibson, *et al.* (1980) examined 160 *Trypanozoon* stocks and revealed that *T. evansi* from South America, Nigeria, Sudan, Kenya and Kuwait formed a homogeneous group in their isoenzyme patterns resembling West African *T. brucei* stocks. The isoenzyme patterns of *T. evansi* collected in Kenya differ from West African *T. brucei* and inclusion of more enzymes could differentiate between *T. evansi* and *T. brucei* stocks (Gibson, Wilson and Moloo, 1983). Limited genetic variations based on the examination of isoenzyme banding patterns were shown by *T. evansi* from widely separated geographical areas:- South America, Nigeria, Sudan, Kuwait, Kenya (Gibson *et al.*, 1980; 1983; Boid, 1988); Brazil (Stevens, Nunes, Lanham *et al.*, 1989) and China (Zheng, Shen, Wang *et al.*, 1990; Lun, Allingham, Brun, *et al.*, 1992a). The isoenzyme patterns of 3 *T. evansi* stocks from Indonesia, however, was reported to have variable patterns similar to that of the rodent-adapted *T. vivax* (Boid and Mleche, 1985).

In the subgenus *Trypanozoon*, isoenzyme analysis has been particularly important in differentiating parasite species which are morphologically indistinguishable (Gibson *et al.*, 1980; Gibson and Wellde, 1985; Godfrey, Baker, Rickman, *et al.*, 1990). Five enzymes; aspartate aminotransferase (ASAT), phosphoglucomutase (PGM), malic enzyme (ME), two peptidases (PEP1 and PEP2) have been used to differentiate cameline *T. evansi* species from others in the *Trypanozoon* subgenus (Gibson *et al.*, 1980).

Apart from simply characterising the various trypanosome species, attempts have also been made to use isoenzymes as markers for drug resistance. Dukes (1984), working with *T. gambiense* sought, without success, to find a correlation between patterns of alanine

aminotransferase (ALAT), ASAT, isocitrate dehydrogenase (ICD), PEP1, PEP2, PGM and ME and resistance to Trypamidium, melarsopol, pentamidine and Berenil. Boid, Jones and Payne (1989) working with Sudanese *T. evansi* stocks suggested that a particular ME pattern (type 7) could be used as marker for *T. evansi.* According to migration on starch electrophoresis gel, ME of *Trypanozoon* parasites has been shown to have 28 variant patterns found in 12 locations (Godfrey *et al.*, 1990). The five isoenzyme types of malic enzyme found in *T. evansi* stocks are patterns 2, 4, 7, 10 and 24 (Gibson *et al.*, 1983; Boid, 1988). The link between suramin resistance and ME pattern 7 has not been observed in stocks from any other parts of the world and its value as a marker remains to be confirmed.

2.3.5. DIVERSITY OF *TRYPANOSOMA* DNA ORGANISATION

The DNA of trypanosomes differs in their characteristics among different species and strains. The DNA of trypanosomes is organised within two organelles, the nucleus and the kinetoplast.

2.3.5.1. Nuclear DNA

With Giemsa's stain, the nucleus is seen as a densely staining mass located at the center or in the anterior half of the body of trypanosome bloodstream forms. The nucleus contains chromatin material, the network of fibres of DNA and protein which stores genetic information needed for all trypanosome life processes.

The nuclear structure of trypanosomes have been studied extensively, particularly in *T. brucei,* which contains approximately 12% highly repetitive DNA; 20% mid-repetitive and some 68% single copy DNA (Borst, Fase-Fowler, Frasch *et al.*, 1980).

The DNA content of the nucleus varies among species, subspecies and strains of trypanosomes. Variations of up to 40% in total DNA as measured by microfluorometry, have been shown between clones and strains of *T. cruzi* (Dvorak, Hall, Crane *et al.*, 1982; Kooy, Ashall, Van der Ploeg *et al.*, 1989). The DNA content of *T.b. gambiense* was reported to be 70% less than *T.b. brucei* and this was suggested to be due to a depletion of the 50-150 kb minichromosomes in *T.b. gambiense* (Dero, Zampetti-Bosseler, Pays *et al.*, 1987). The DNA content of *T. evansi* is reported to contain 0.2 pg per nucleus (Baker, 1961) which is significantly higher than the 0.097 pg per nucleus found in the related *T. brucei* (Borst *et al.*, 1980). The discrepancy in DNA content of these closely related subspecies was suggested to be due to the different measurement technique employed (Borst *et al.*, 1980).

In common with other protozoan parasites the chromosomes of trypanosomes do not condense at any stage of the cell cycle (Vickerman and Preston, 1970). The absence of chromosome condensation makes it impossible to carry out a conventional microscopic

analysis of their karyotypes. Pulsed-field gel electrophoresis (PFGE) (Schwartz and Cantor, 1984) has been used to estimate the amount of nuclear DNA per haploid genome for *Leishmania* as between 23 Mb in *L. panamensis* (Scholler, Myler and Stuart, 1989) to 67 Mb in *L. mexicana* (Galindo and Ramirez Ochoa, 1989). The haploid genome size of *T. brucei* has been estimated by Myler (1993) to be approximately 30-40 Mb on the basis of renaturation analysis carried out by Borst *et al.* (1980). Borst *et al.* (1980) estimated that the 68% of single copy DNA consisted of $2.5x10^7$ base pairs. The numerical values of chromosome sizes add up to more than 60 Mb in *T. brucei* (Gottesdiener, Garcia-Anoveros, Lee *et al.*, 1990) and 87 Mb in *T. cruzi* (Cano, Gruber, Vasquez *et al.*, 1995; Kooy *et al.*, 1989).

2.3.5.2. The Kinetoplast

The diameter of the kinetoplast ranges from 0.4 µm in the subgenus *Pycnomonas* to 1.4 µm in the subgenus *Duttonella* (Hoare, 1972). The kinetoplast is equivalent to the mitochondria of other cells and contains extra-nuclear chromatin material which is important in coding for the enzymes used for energy production (Hoare, 1972). Its sizes complexity and location varies according to both the trypanosome species and the life cycle stage (Vickerman, 1970).

Some trypanosome stocks appear to lack a kinetoplast when seen under the light microscope. These dyskinetoplastic forms have been shown by electron microscopy studies to have the membranous envelope commonly found around this organ but to lack the central chromatin DNA material (Vickerman, 1963). Dyskinetoplasty has been shown to be an induced mutation that is inherited as trypanosomes reproduce. The highest proportion of dyskinetoplasty occurs in the subgenus *Trypanozoon*, especially in *T. evansi.* In this species, the occurrence of dyskinetoplastic forms is best exemplified by the two South American strains, *T. venezuelense* and *T. equinum,* which either have a high proportion of dyskinetoplastic forms or totally lack a visible kinetoplast. Since both these strains conform to the classical *T. evansi* identity in other respects, both *T. venezuelenze* and *T. equinum* are considered mutant aberrant forms and not as separate species (Hoare, 1972).

The presence of a kinetoplast DNA (kDNA) network is a characteristic of all members of the order Kinetoplastida including trypanosomes. The structure of kDNA is a network of semi-random catenation of minicircles that are constrained in a flat, round disc and maxicircles are catenated into the minicircle network (Reviewed by Englund, Hajduk and Marini, 1982; Borst and Hoeijmakers, 1979 a; b; Stuart, 1983). Further studies showed that the structure of kDNA in trypanosomatids is condensed by catenation rather than supercoiling (Rauch, Perez-Morga, Cozzarelli *et al.*, 1993) which is in contrast to that reported in *T. equiperdum* (Riou and Saucier, 1979). The kDNA network contains 5,000-10,000 minicircles,

representing >95% of the network mass and 25-50 maxicircles. The DNA sequence of minicircles vary within species, except of *T. evansi* whose minicircles are homogeneous (Songa, Paindavoine, Wittouck, *et al.*, 1990). *Trypanosoma evansi* however, does not possess maxicircles (Borst and Hoeijmakers, 1979a; Borst, Fase-Fowler and Gibson, 1987). The variety of kinetoplast DNA properties is presented in **Table 2.2**.

Table 2.2. Properties of kinetoplast DNA (Adapted from Englund, 1981).

	Network molecular weight x 10^{-10}	Maxicircle size (kb)	Minicircle size (kb)	Minicircle sequence classes*)
T. brucei	0.40	20-22	1.0	~300
T. mega	Uk	26.0	2.3	70
T. cruzi	2.10	33.0	1.44	<20
C. fasciculata	0.72	38.0	2.5	10-20
C. luciliae	2.20	38.0	2.5	10-20
P. davidi	-	39.0	1.06	<10
L. tarentolae	0.60	31.0	0.87	3
T. equiperdum	0.36	14.3-23.0	1.0	1
T. evansi	UK	ND	1.0	1

*) These values are the approximate number of the major minicircle sequence classes. Some values are estimates (Englund, 1981).
ND = Not detected
UK = Unknown

Minicircles

Because of their small circular size and abundance in the kDNA network the minicircles in trypanosomes are unique mitochondrial DNA. The minicircles are responsible for the structure of the kDNA network (Stuart, 1983). Each network contains thousands of minicircles. The kinetoplast DNA minicircles comprise of 5-25% of the total DNA (Simpson, 1986; Stuart, 1983; Englund *et al.*, 1982) and are constant in size within species. However, among different species of kinetoplastid flagellates the sizes of the minicircles vary from 0.5 kb in *T. vivax* (Borst, Fase-Fowler, Weijers *et al.*, 1985) to 2.5 kb in *Crithidia fasciculata* (Sugisaki and Ray, 1987).

The only known function of minicircles in trypanosomes is to encode guide RNA (gRNA) (Sturm and Simpson, 1990), which mediates in the editing of maxicircle transcripts (Blum, Bakalara, Simpson, 1990; Benne, 1990; Sturm and Simpson, 1990; 1991; Pollard and Hajduk, 1991; Pollard, Rohrer, Michelotti, *et al.*, 1990; Van der Spek, Arts, Zwaal, *et al.*,

1991). Since *T. evansi* does not possess maxicircles (Borst *et al.*, 1987) the function of minicircles in *T. evansi* remains uncertain. Gajendran, Vanhecke, Songa *et al.* (1992) suggested that the minicircles of kDNA in *T. evansi* encode gRNA genes. Why *T. evansi* maintains the gRNA gene sequences in the absence of maxicircles is not known; however gRNA genes might be used for genes other than for editing the maxicircle transcripts (Gajendran *et al.*, 1992).

The minicircle organisation in trypanosomes generally has a common genomic organisation, consisting of a small conserved region, which is maintained in all minicircles and a large variable region, which differs between minicircles (Simpson, 1987). The conserved region differs in size and copy number in different species. *Trypanosoma evansi* (Ou, Giroud, Baltz, 1991), *T. brucei* (Chen and Donelson, 1980; Jasmer and Stuart, 1986), *T. equiperdum* (Barrois, Riou, Galibert, 1981), *T. congolense* (Nasir, Cook and Donelson, 1987) have a single conserved region of 130-150 bp in each 1 kb minicircle. The conserved region present in the minicircle of *T. evansi* is almost homologous to that of *T. equiperdum* and *T. brucei* (Songa *et al.*, 1990). *Trypanosoma lewisi* has two conserved regions of two direct repeats of 95 bp in 1 kb minicircle (Ponzi, Birago and Battaglia, 1984; Zhang and Deisseroth, 1991) and *T. cruzi* has four regions of 118 bp in a 1.4 kb minicircle (Macina, Sanchez, Affranchino *et al.*, 1985; Ryan, Shapiro, Rauch *et al*, 1988).

Intra-species sequence heterogeneity in minicircles is found in *T. brucei* (Chen and Donelson, 1980). The most extreme heterogeneity in the minicircle sequences is found in *T. brucei* with the presence of ~300 sequence classes (Stuart and Gelvin, 1980; Stuart, 1983). *Trypanosoma evansi* (Borst *et al.*, 1987; Songa *et al.*, 1990; Ou *et al.*, 1991; Lun, Brun and Gibson, 1992b; Artama, Agey and Donelson, 1992) and *T. equiperdum* (Barrois *et al.*, 1981) were reported to have homogeneous minicircle sequences. Homogeneity in the minicircle sequences was reported in *T. evansi* stocks from China, the Philippines, Ethiopia (Ou *et al.*, 1991), Africa and South America (Lun *et al.* 1992b). Sequence homology was also noted in the *T. evansi* stocks from Thailand, Nigeria, Kenya, Sudan, Colombia (Songa *et al.*, 1990) and Indonesia (Songa *et al.*, 1990; Artama *et al.*, 1992). This sequence homogeneity of the minicircle kDNA of *T. evansi* supports the hypothesis of a single origin for *T. evansi* isolates as has been suggested by isoenzyme analysis (Gibson *et al.*, 1983; Boid, 1988).

Despite the reports of homogeneity in the minicircle sequences of *T. evansi*, Borst *et al.* (1987) detected a "type B" minicircle in a *T. evansi* stock isolated from East Africa, which differed from those detected in the rest of *T. evansi* stocks studied ("Type A"). Ou *et al.* (1991) demonstrated that the minicircles of 4 kinetoplastic clones of *T. evansi* were not fully homogeneous but had limited base sequence diversity. Sequence variation in the "variable region" of minicircles of *T. evansi* can be used to distinguish *T. evansi* from other member of *Trypanozoon* (Masiga and Gibson, 1990).

2.3.6. GENE DIVERSITY

Gene diversity in trypanosomes occurs through either sexual or asexual reproduction. Sexual reproduction that occurs in trypanosomes, such as *T. brucei*, leads to a much greater diversity in the population. Less gene diversity is expected to occur when there is no sexual reproduction. As the presence of the sexual cycle in *T. evansi* is not known, the gene diversity in this parasite could occur through genetic rearrangements such as mutation, inter- and intrachromosomal recombination.

Isoenzymes and antigenic analysis have been the most common characteristics used to study gene variation. Enzyme electrophoresis has been used to assess genetic relationships both within and between species (Gibson *et al.*, 1983; Stevens and Godfrey, 1992), to determine the ploidy of an organism (Tait, 1980) and to investigate population structures (Tibayrenc and Ayala, 1991).

The presence of a diploid genome in *T. brucei* has been shown by quantitative measurements of nuclear DNA content and complexity (Borst, Van der Ploeg, Van Hoek, *et al.*, 1982); analysis of restriction site polymorphisms in housekeeping genes (Gibson *et al.*, 1985; Tait, Turner, Le Page *et al.*, 1989) and genetic analysis (Tait, 1980; Tibayrenc, Ward, Moya, *et al.*, 1986). On the contrary, Borst and Cross (1982) suggested haploidy in *T. brucei* genome on the basis of measurement of the copy number of specific variant antigen gene. The detection of haploidy in *T. brucei* by Borst and Cross (1982) might be due to the presence of two copies of an antigen gene which are located at identical positions of homologous chromosomes (Tait and Turner, 1990). Haploidy in *T. brucei* was also reported by Zampetti-Bosseler, Schweizer, Pays *et al.* (1986) who detected half of the DNA content in the metacyclic stage compared to that in bloodstream or procyclic stages. However, Tait and Turner (1990) argued this conclusion by presenting the fact that extensive analysis of the DNA contents of both metacyclic and bloodstream stages of *T. brucei*, *T. congolense*, *T. vivax* and *T. simiae* using similar methods (Kooy *et al.*, 1989) showed identical DNA content, and therefore have the same ploidy.

It was also shown that genetic exchange could occur between different strains of *T. brucei* in tsetse fly (Jenni *et al.*, 1986; Tait and Turner, 1990). Genetic exchange also occurs in natural populations of *T. brucei* as has been shown by isoenzyme surveys (Gibson *et al.*, 1980; Tait, 1980; 1983; Cibulkis, 1988). The evidence of genetic exchange was first suggested by Jenni *et al.* (1986) in *T. brucei*; then by Paindavoine *et al.* (1986a); Sternberg, Tait, Haley *et al.* (1989) and Gibson (1989).

Studies have been carried out to determine if genetic exchange occurs within *T. brucei* subspecies. Paindavoine *et al.* (1989) concluded that there was no evidence to support that genetic exchange occurs between *T.b. brucei* and *T.b. gambiense*, which might be due to

T.b. gambiense being genetically different from other sympatric subspecies. Genetic exchange occurred between *T.b. brucei* and *T.b. rhodesiense* based on the observation of co-transmission of both subspecies through tsetse flies and PFGE analysis of the resultant trypanosome population which revealed the presence of the non-parental karyotypes (Gibson, 1989). Each of the progeny clones had at least one genetic characteristic from each parent which provides good evidence to indicate that *T. brucei* is diploid (Gibson, 1989). The presence of genetic exchange in *T. evansi* has not been established since the relationship of *T. evansi* with any vector is unknown.

2.3.7. DRUG RESISTANCE

Characteristics such as trypanosome length, isoenzyme patterns and kDNA analysis are important in determining morphological, biological, genetic and epidemiological features. The drug-sensitivity of trypanosome isolates is however, a characteristic of greater practical importance.

Drug resistance has been described as the ability of trypanosomes previously exposed to a drug, to survive despite the administration of that drug at dose rates equal to or higher than those usually recommended (Schillinger, 1985). Drug resistance can be heritable (Geary, Edgar, Jenson, 1986), which means its development may involve DNA mutations that can be inherited from one generation to the other. Drug resistance can also be caused by non-genetic changes (Georgopoulos, 1982) in the target cells as a result of phenotypic adaptation due to the prolonged exposure of a drug. Natural resistance to trypanocidal drugs had been observed (Leach and Roberts, 1981) in conditions where trypanosomes had never been exposed to the drugs. The evidence of natural resistance was observed by the differences in trypanocidal efficacy in various subgenera, species, subspecies (Lumsden and Ketteridge, 1979) or even strains derived from a single clone (Peregrine, Knowles, Ibitayo *et al.*, 1980).

Drug resistance to trypanosomes has usually been attributed to the occurrence of relapse infections after drug treatment under conditions that excluded reinfection (Jennings, Whitelaw and Urquhart, 1977). Schillinger (1985) pointed out several factors causing failures in the chemotherapy of trypanosomiasis: depressed immune status by the host; evasion of trypanosomes to different target organs unsusceptible to the drug used; drug tolerance (natural resistance) and drug resistance (induced resistance).

Control of surra relies on the treatment of infected animals since the vectors are not known with certainty and current fly control strategies are inefficient. A limited number of trypanocidal drugs are available and these have been used extensively resulting in the appearance of trypanosome strains resistant to these drugs. *Trypanosoma evansi* strains

resistant to suramin have been reported in India (Gill, 1971), Sudan (Luckins, Boid, Rae *et al.*, 1979; Boid *et al.*, 1989), former USSR (Petrovski and Khamiev, 1974), Kenya (Gitatha 1980; Schillinger, Moloo and Rottcher, 1985), and Indonesia (Sukanto, Agustini, Stevenson *et al.*, 1990). Suramin is still the drug of choice to treat surra in Indonesia (Sukanto *et al.*, 1990; Payne, Sukanto, Partoutomo *et al.*, 1994a) because widespread resistance exists to isometamidium (Sukanto *et al.*, 1990), the alternative trypanocidal drug available in the country.

Treatment of trypanosome infections sometimes fails in hosts whose immune responses had been depressed, by malnutrition, work-stress or infection with other parasites. Laboratory experiments have shown the rapid development of drug resistance to mel Cy (Cymelarsan, Rhone Merieux), diminazene aceturate and isometamidium chloride (Samorin, RMB, Dagenham, UK) in immunosuppressed mice infected with *T. evansi* (Osman, Jennings and Holmes, 1992). Studies carried out on the treatment of infected animals with trypanostatic drugs, such as DL-α-difluoromethylornithine (DFMO), showed that these drugs require a fully competent immune system to eliminate the parasite (deGee, Mc Cann and Mansfield, 1983). Immunosuppression due to infection with other parasites, malnutrition or work-stress is common in animals in the tropics (Schillinger, 1985; Clemence, 1997 Ph.D. thesis).

Trypanosomes have an ability to invade the body compartments other than the vascular system or in an unsusceptible developmental stage, which usually occurs at the late stage of an infection. *T. brucei* persists in the tissue as the amastigote form which is unsusceptible to treatment and will eventually cause a reappearance of the bloodstream forms (Jennings *et al.*, 1977). *Trypanosoma evansi* invades the central nervous system, even within 2 weeks of infection (Kraneveld and Djaenoedin, 1949) and therefore evades most of the available drugs that cannot cross the blood-brain barrier. This phenomenon has been described in diminazene aceturate (Jennings *et al.*, 1977) and homidium bromide (Dolan, Okech, Alushula *et al.*, 1990) treated animals.

Drug tolerance is a natural resistance in which parasites have the ability to tolerate certain trypanocides without previous contact with the drug (Leach and Roberts, 1981). Isometamidium was only introduced into Indonesia in 1977 (Soekardono, 1977) but widespread isometamidium resistance has already been reported (Sukanto *et al.*, 1988; Payne, Waltner-Toews, Djauhari *et al.*, 1991b). An attempt to control an outbreak of trypanosomiasis in Madura, an area the drug had never been used in before, was unsuccessful (Sukanto *et al.*, 1988; Payne *et al.*, 1990).

Drug resistance can be reduced by passages of a resistant strain through different host species, however, if the parasite is returned to the original host species, resistance returns (van Zwieten, 1932). This implies that transmission of trypanosomes by fly vectors should

make a strain more sensitive. In her review, Dieleman (1983) did not find any reports of suramin resistant field *T. evansi* stocks in Indonesia. Several years later 3/27 *T. evansi* field stocks was found to be resistant to suramin at the dose rate of 5 to 10 mg/kg body weight (Sukanto *et al.*, 1990).

Various sensitivities to trypanocidal drugs were observed in different trypanosome species. *Trypanosoma vivax* stocks isolated from West Africa were reported to be more susceptible to homidium than were *T. congolense* from East Africa (Scott and Pegram, 1974), however *T. congolense* was more susceptible to diminazene than *T. vivax* (Williamson, 1970). Peregrine (1994) in his review mentioned different trypanosome species that are resistant to various trypanocides used to treat cattle in 11 countries in west, central, east and southern Africa.

Differences in sensitivity to trypanocidal drugs were reported in various *T. evansi* stocks from different geographical origin. *Trypanosoma evansi* strains from China was reported to be resistant to diminazene aceturate (Berenil, Hoechst AG, FRG) (Zhang, Giroud and Baltz, 1993). The *T. evansi* stocks were isolated from southern China where Berenil has been used for more than 30 years. In Indonesia, Diminazene is partially effective against surra (Sukanto *et al.* 1990) with a 50% efficacy in *T. evansi* stocks tested in a sensitivity test in mice. However, Holz and Adiwinata (1956) failed to cure *T. evansi* infection in horses with Berenil in Indonesia. *Trypanosoma evansi* stocks from India were reported to be sensitive to Berenil (Gill and Sen, 1971; Hiregoudar and Avsatthi, 1971).

Trypanosoma evansi stocks from China were reported to be sensitive to Cymelarsan (Lun, Min, Huang *et al.*, 1991), however *T. evansi* stocks from Indonesia were less susceptible. It was demonstrated (Payne, Sukanto, Partoutomo *et al.*, 1994b; 1994c) that a higher dose rate of Cymelarsan (0.75 mg/kg bodyweight instead of 0.25 mg/kg bodyweight used in China) is required to eliminate *T. evansi* infection in buffaloes in Indonesia.

It was also observed that *T. brucei* stocks resistant to diminazene were less susceptible to cymelarsan (Zweygarth and Kaminsky, 1990; Zweygarth, Ngeranwa and Kaminsky, 1992; Frommel and Balber, 1987). However, *T. evansi* and *T. equiperdum* stocks from China that are resistant to berenil, suramin, quinapyramine or isometamidium were fully sensitive to cymelarsan (Zhang, Giroud and Baltz, 1992; 1993).

The sensitivity of trypanosome to a drug ranges from complete sensitivity where all trypanosomes are killed, to complete resistance where the drug has no effect at all on the infection (Schillinger, 1985). Some strains of *T. evansi* in Indonesia became resistant as a result of treating animals with subcurative doses of drugs when the need for prophylactic treatment was not certain (van Zwieten, 1932).

Several methods have been applied to detect resistance including *in vivo* in lab animals (Rottcher and Heising, 1981), *in vitro* (Sutherland, Taylor and Ross, 1993; Sutherland, Barns and Ross, 1995). Boid *et al.* (1989) found that malic enzyme type VII can be used in Sudanese stocks of *T. evansi* as a marker of suramin resistance. However, the relationship between the presence of this enzyme and suramin resistance has not been confirmed in subsequent studies (Boid, Hunter, Jones *et al.*, 1996). Determination of the distribution of drug resistance is important for the application of trypanosome control strategies in endemic areas.

2.4. TECHNIQUES FOR MOLECULAR CHARACTERISATION OF KINETOPLASTIDS

2.4.1. INTRODUCTION

The ability to identify different trypanosome characteristics should contribute to a better understanding of the epidemiology of the parasite and in the establishment of effective disease control programmes.

The most specific method of characterising trypanosome species must lie at the genetic level. Molecular biological methods rely on the fact that each species has characteristic DNA that distinguishes it from all other species. These variations in DNA might be expressed as identifiable phenotypic characteristics such as variations in infectivity to vertebrate hosts, morphology and life cycles. DNA techniques offer the advantage of directly analysing trypanosome DNA instead of older, indirect methods that detect phenotypic characteristics resulting from changes in the DNA.

2.4.2. MOLECULAR-BASED TECHNIQUES

2.4.2.1. Pulse Field Gel Electrophoresis of DNA

Gel electrophoresis is a standard technique for characterising DNA fragments, however, several parameters affect the quality of separation and mobility of DNA molecules in gel electrophoresis including the type and concentration of the gel, the temperature, voltage gradient and the composition of the buffer. In conventional gel electrophoresis the separation of molecules depends mainly on the sieving properties of the gel matrix in which smaller fragments move through the gel matrix more easily, and thus faster, than the larger ones (**Figure 2.1A**). In conventional gel electrophoresis the size of DNA that can be separated has an upper limit of 15 to 20 kb (Fangman, 1978) but larger molecules up to 50

kb can be separated under special conditions (de Gennes, 1971; Lerman and Frisch, 1982; Lumpkin, Dejardin and Zimm, 1985). Under ordinary electrophoresis conditions, however, all molecules larger than 20 kb will move at the same rate in a static electric field and the DNA fragments are not separated from each other. Past attempts to separate large DNA molecules by electrophoresis using low gel concentration and low voltage gradient were not successful (Serwer, 1981) because low percentage agarose gels are mechanically difficult to handle and the use of low voltage gradient requires long running times.

In 1982, Schwartz and his co-workers offered a solution to the problem of separating DNA molecules larger than 50 kb by using two alternating electric fields that were not homogeneous thereby introducing the technique of pulsed-field gel electrophoresis (PFGE) (Schwartz, Saffran, Welsh *et al.*, 1982). Schwartz and Cantor (1984) showed that the use of non-uniform electrical fields was critical in achieving high resolution. It was suggested (Schwartz and Cantor, 1984) that an electrical field gradient would stretch the molecule of a DNA coil. Furthermore, the use of inhomogeneous electrical fields in pulse field gel electrophoresis improves resolution of individual components due to the net decrease of the field in the direction of migration of each DNA band. The leading edge of each DNA band moves slower than the trailing edge, because the field gradient decreases, resulting in a more compact band (Schwartz and Cantor, 1984).

In PFGE, the DNA molecules must first elongate themselves in the direction of the first field (F1 in **Figure 2.1B**) before they can begin to migrate through the gel. The first electric field is then removed and a second field (F2 in **Figure 2.1B**), which located at an angle to the first field, is activated. The DNA must change conformation and reorient before it can start to migrate in the direction of the second electric field. The time required for this reorientation has been found to be related to the length of the molecule. Larger DNA molecules take more time to realign after the field is switched than smaller ones because of the physical barrier of the agarose matrix. Hence, molecules of increasing size must spend a larger portion of each switching cycle reorienting before they can begin to migrate through the gel. Because of the additional electric fields involved in PFGE, a number of terms had been introduced to describe the electrophoretic conditions in use (**Table 2.3**).

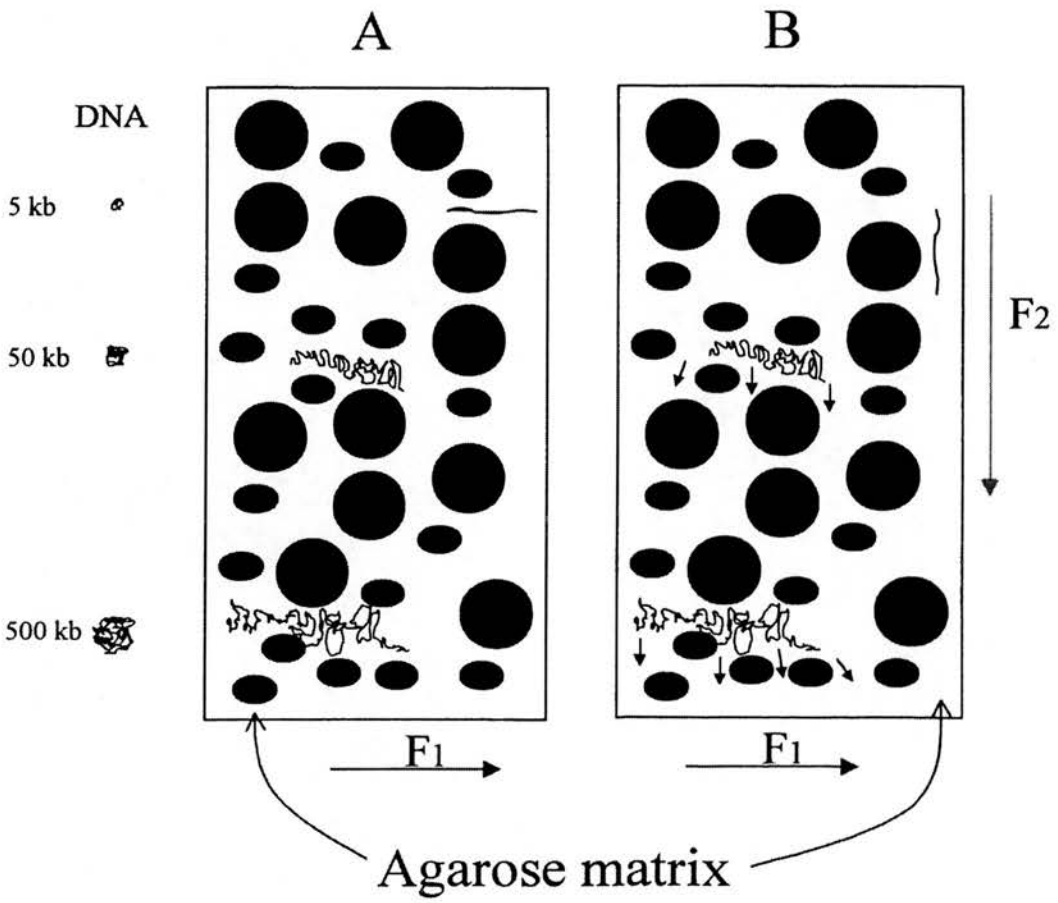


Figure 2.1. Schematic illustration of DNA separation in Conventional (A) and Pulsed Field (B) Electrophoresis

Table 2.3. Terms used in PFGE

Pulsed field	An electrophoresis procedure that uses multiple electric fields and in which the electric fields are activated in an alternating order.
Switch interval (switch time, pulse time)	Length of time each of the alternating electric fields is active.
Reorientation angle	The angle between the two alternating electric fields.
Field inversion	PFGE system in which the two alternating fields have a 180° reorientation angle.
Voltage gradient	Electrical potential applied across the gel (volts/ cm)
Homogeneous field	Electrical field that has a uniform potential difference across the whole field.

The original term pulsed field gradient gel electrophoresis (PFGE) was applied by Schwartz and Cantor (1984) to any gel run using alternating multiple electric fields. It is now clear that a field gradient is neither an important nor a desirable aspect of pulsed field gels. Using the field gradient resulted in DNA molecules of the same size moving at different rate depending on their location in the gel and resulted in a curved trajectory in the gel and making lane to lane comparisons and size estimations for the DNA bands difficult (Birren and Lai, 1993). The abbreviation PFG is now used to imply homogeneous pulsed field gel and not pulsed field gradient. Subsequently, other names (e.g., OFAGE, FIGE, TAFE, CHEF, PACE, RGE, crossed-field electrophoresis, ST/RIDE) have been given to pulsed field electrophoresis systems that involve variations on the original electrode geometry, homogeneity, and method of reorientation of the electric fields (**Table 2.4**). Most of these names are used to describe a particular hardware design (e.g., electrode geometry, electrical circuit), not a specific mechanism for the separation.

These different systems rely on the same principle for separation, that is, they subject the DNA molecules to at least two alternately active electric fields. The maximum DNA size limit that can be resolved does not seem to differ among these systems. The major differences among the various systems are (1) whether straight lanes can be obtained; (2) the speed of separation; (3) the resolution within a particular size range; and (4) the portion of the gel that provides usable separation.

Table 2.4. Pulsed Field Gel Acronyms

Acronym	Electrophoresis systems	Reference
PFGE	Pulsed field gradient electrophoresis	Schwartz and Cantor (1984)
OFAGE	Orthogonal field alternation gel electrophoresis	Carle and Olson (1984)
TAFE	Transverse alternating field electrophoresis	Gardiner *et al.* (1986)
FIGE	Field inversion gel electrophoresis	Carle *et al.* (1986)
CHEF	Contour clamped homogeneous electric field	Chu *et al.* (1986)
RGE	Rotating gel electrophoresis Cross field gel electrophoresis	Serwer (1987) Southern *et al.* (1987)
PHOGE	Pulsed homogeneous orthogonal field gel electrophoresis	Bancroft and Wolk (1988)
PACE	Programmable autonomously controlled electrodes	Clark *et al.* (1988)
ST/RIDE	Simultaneous tangential/rectangular inversion decussate electrophoresis	Kolble and Sim (1991)

Several apparatuses have been developed for separating large DNA molecules (**Table 2.4**). The common feature of these systems is the use of multiple electric fields. All pulsed field gel electrophoresis systems can separate DNA in the same size range but differ in the speed of separation and the resolution obtained. The main features of each system are summarised in **Table 2.5**.

Table 2.5 Comparison of PFGE Systems

Characteristics	SYSTEM						
	PFGE/ OFAGE	TAFE	FIGE	CHEF	RGE	ST/ RIDE	PACE
Produces straight lanes	No	Yes	Yes	Yes			
Homogeneous electric fields	No	Yes	Yes	Yes			
Multiple fields	No	No	No	No			
Resolution							
1-50 kb	+	+	++++	+	No	+	++++
50 –500 kb	+	++++	+	+	++	+	++
2000 –7000 kb	+		+	+	++	+	++
Constant angle across gel	No	No	Yes	Yes	Yes	Yes	Yes
Variable reorientation angle	No	No	No	No	Yes	Yes	Yes

Note: Separation ranged from adequate (+) to very good (++++)

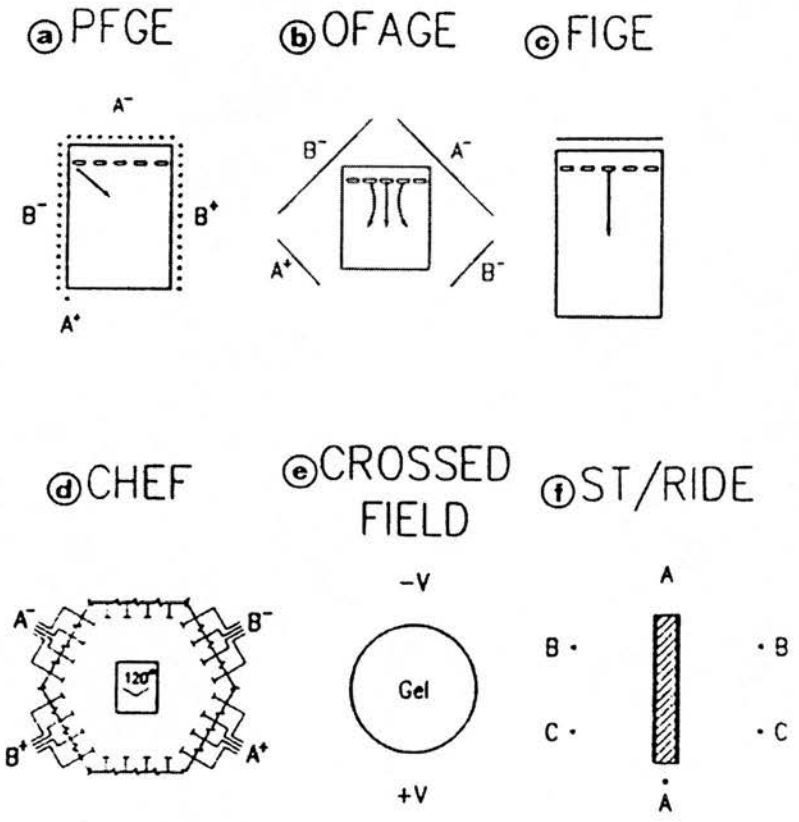


Figure 2.2. Schematic diagram of pulsed field gel electrophoresis systems.

Schwartz and Cantor (1984) introduced a PFGE technique that used two alternating electric fields, one homogeneous and the other nonhomogeneous (**Figure 2.2a**). This technique was effective in separating *Saccharomyces cerevisiae* chromosomes in the size range of 200 kb to 3 Mb length (Schwartz and Cantor, 1984). This PFGE technique had been used by Van der Ploeg, Schwartz, Cantor, *et al.* (1984a) to analyse antigenic variation in *T. brucei* and found that changes in the intermediate-sized chromosomes (200-700 kb) were associated with changes in the surface antigen genes expression. Using PFGE, Gibson and Borst (1986) found that both *Trypanozoon* and *Nannomonas* have the same basic organisation of chromosomes, which consisted of large (megabase size chromosomes), intermediate (150-650 kb) and small chromosomes (50-150 kb).

Carle and Olson (1984) developed a similar apparatus using two nonhomogeneous electric fields, orthogonal-field-alternation gel electrophoresis (OFAGE) (**Figure 2.2b**). In OFAGE the DNA fragments are separated by alternately applying two electric fields that are approximately orthogonal. The OFAGE had been used to study the chromosome profiles of *T. congolense* isolates from Kenya (Masake, Nyambati, Nantulya *et al.*, 1988) and showed stability in the karyotype patterns between 100-1,000 kb size range of stocks after passage to different host species. The disadvantage of both PFGE and OFAGE is that DNA molecules migrate at different rates dependent on their location in the gel resulting in bent lanes, which causes problems when comparing results from lane to lane in the gels.

Field inversion gel electrophoresis (FIGE) developed by Carle, Frank and Olson (1986) is based on the separation of large DNA molecules by periodically inverting a uniform electric field in one dimension, that is, using a 180^0 reorientation angle (**Figure 2.2c**). This technique is good at resolving DNA molecules smaller than 750 kb (Carle *et al.*, 1986, Lai, Birren, Clark *et al.*, 1989). The disadvantage of this system is that DNA fragments of different sizes can migrate with the same mobility (Lai *et al.*, 1989) as a property of the FIGE system which causes band inversion (Carle and Olson, 1987).

Contour clamped homogeneous electric field electrophoresis (CHEF) developed by Chu, Vollrath and Davis (1986) uses twenty-four electrodes arranged in a hexagonal contour, to produce reorientation angles of between 60^0 or 120^0 (**Figure 2.2d**). The CHEF system has been used to separate chromosomal DNA of different species of parasite. This technique was used to separate chromosomal DNA of *T. evansi* in the size range of 50-600 kb (Waitumbi and Young, 1994) and between 200-1,100 kb (Lun *et al.*, 1992b). The CHEF system was used by Gibson and Bailey (1994) and Gibson, Kanmogne and Bailey (1995) to separate chromosomal DNA of *T. brucei* between 250 kb to >2 Mb size range and showed that most clones derived from two parental clones of *T. brucei* inherited the karyotype from each parent as judged by the size of their chromosomal bands. CHEF was also used for separating chromosomes of 15 species of *Leishmania* between 200-2,000 kb size range

(Cruz Tavares, Grimaldi and Traub-Cseko, 1992). Using the same system Beja, Schwartz and Michaeli (1994) separated 31 distinct chromosomal bands between 250-1650 kb size range in *Leptomonas collosoma*. The major advantage of the CHEF system is the ability of the system to generate straight lanes in large numbers of DNA samples subjected to the system.

It was suggested (Birren and Lai, 1993) that similar DNA separation to that of the CHEF system could also be obtained using the cross-field gel electrophoresis (Southern, Anand, Brown, *et al.*, 1987) and rotating field gel electrophoresis (RGE) (Serwer, 1987) apparatus (**Figure 2.2e**). The DNA molecules move in a straight lane due to the homogeneous fields. The system was shown to give sharp resolution of the DNA fragments of up to and greater than 10 Mb (Southern *et al.*, 1987). Cross-field electrophoresis was used for molecular karyotype characterisation of different species of *Leishmania* between 220-950 kb size range (Katakura, Matsumoto, Gomez *et al.*, 1993). The only disadvantage of this system is the inability to switch the field orientation quickly enough to separate DNA molecules smaller than 50 kb (Lai *et al.*, 1989).

The programmable autonomously controlled electrodes (PACE) system developed by Clark, Lai, Birren, *et al.* (1988) is similar to the CHEF system, but in PACE the voltage of each electrode is independently and actively controlled by separate digital-analogue converter (DAC) circuits. The advantage of PACE is the ability of the system to generate an unlimited number of electric fields of controlled homogeneity, voltage gradient, orientation and running duration. Lai *et al.* (1989) suggested that the PACE system could perform all previous PFGE switching regimens (FIGE, OFAGE, PHOGE and cross-field gel electrophoresis).

Simultaneous tangential/Rectangular inversion decussate electrophoresis (ST/RIDE) was developed by Kolble and Sim (1991) (**Figure 2.2f**). The ST/RIDE used a gel box similar to that of the TAFE system with an additional pair of electrodes in the same plane as the vertical gel. Because of the geometry of the electrode in the ST/RIDE system, band stacking that present in the bottom of the gel in TAFE system should be minimised.

The TAFE system (**Figure 2.3**) was developed (Gardiner, Laas, Patterson, 1986) as a modification of the PFGE system developed by Schwartz and Cantor (1984). In the TAFE system the DNA moves straight in all lanes (Gardiner, 1992). The DNA fragments separated by TAFE system are compressed and highly resolved and well resolved and sharp in DNA fragments smaller than 1,600 kb and the separation is obtained in relatively short electrophoresis time (< 18 hr); however longer electrophoresis time (3-6 days) is required to resolve larger DNA molecules (the megabase size fragments) (Gardiner, 1992).

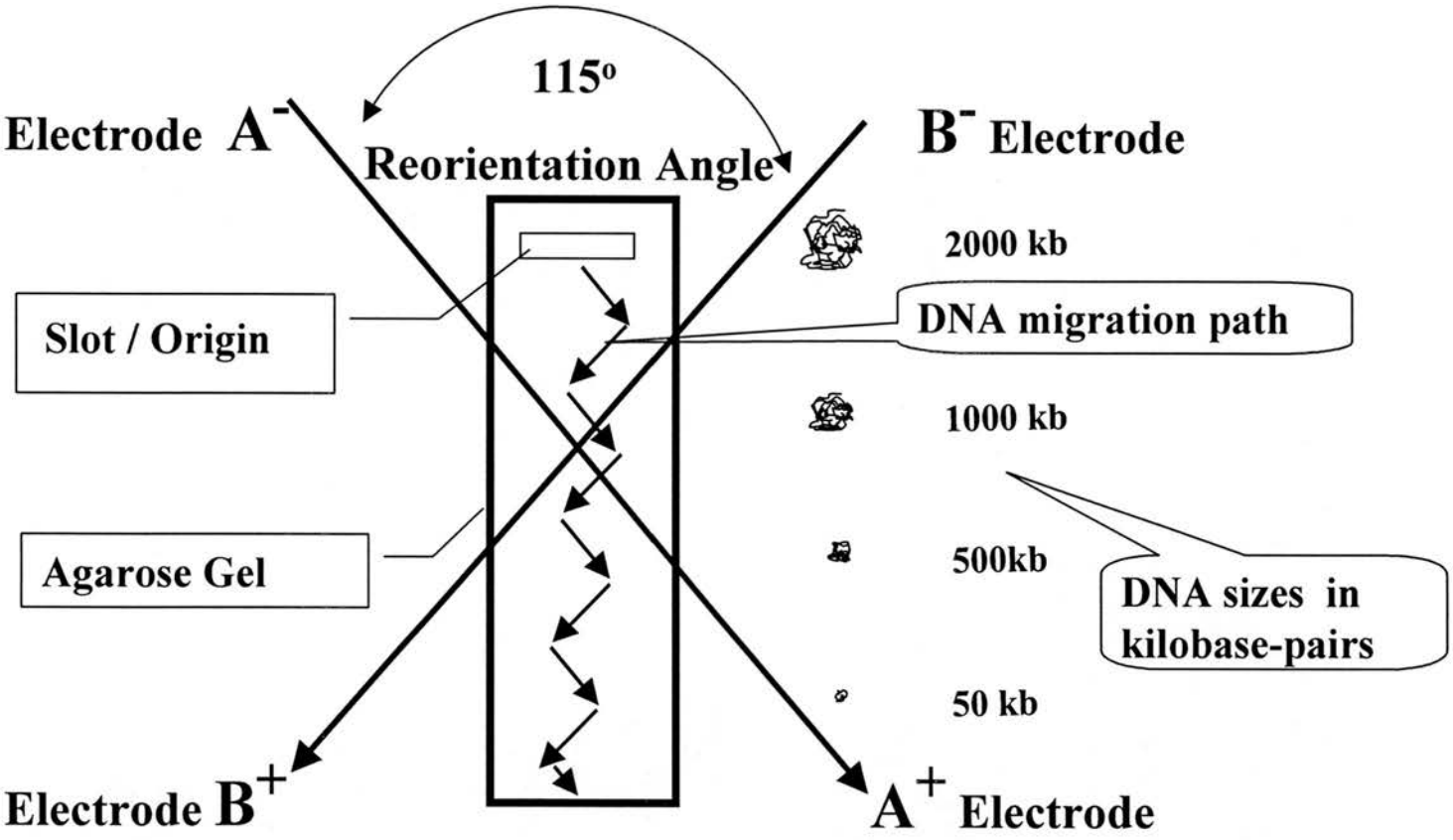


Figure 2.3: Transverse Alternating Field Electrophoresis

The disadvantage of using TAFE is that molecules do not migrate at a constant velocity over the length of the gel due to the variation in the reorientation angle from the top to the bottom of the gel (Lai *et al.*, 1989). The reorientation angle is 115^0 at the top of the gel where the wells are located, but it almost 165^0 at the bottom of the gel (Gardiner, 1992; Lai *et al.*, 1989). Consequently the DNA moves slower near the bottom of the gel as the reorientation angle is increased which leads to poor resolution and stacking of the smallest DNA fragments (Birren and Lai, 1993; Gardiner and Patterson, 1989; Lai *et al.*, 1989).

A commercial apparatus using the TAFE system is available from Beckman, the GeneLine™ II, which consists of an electrophoresis tank incorporated with buffer circulation system. The system allows control of both electric field and buffer environment to produce sharp, well-resolved and reproducible bands in every electrophoresis run. The Controller in the GeneLine™ apparatus automatically regulates the total run time, pulse time and electrical parameters for one or more stages. Low buffer temperatures are held constant by the buffer cooler throughout the electrophoresis run, the pump circulates cooled buffer around the gel throughout the run and after electrophoresis until the gel is removed. In this system the angle between fields is not altered, that is, the reorientation angle is fixed at 115^0. The sharp DNA fragments near the bottom of the gel are produced by the changes in electric field down the gel. The TAFE system was used to separate chromosomal DNA between 350-1,000 kb size range of *Leishmania major* (Giannini, Curry, Tesh *et al.*, 1990) and showed stability in the chromosomal banding pattern upon passage through sandflies and mice. Orozco, Baez-Camargo, Gamboa *et al.* (1993) used TAFE to separate 11 to 20 chromosomal bands of *Entamoeba histolytica* between the size range of 30-3,000 kb and showed differences in the DNA organisation of genetically related clones suggesting a high variety in the genotypes and phenotypes of *E. histolytica*. TAFE was also used to separate chromosomal DNA of *T. evansi* stocks from Indonesia which showed polymorphism in their karyotype patterns (Boid, Jones, Payne *et al.*, 1992; Sukanto, Payne, Boid *et al.*, 1992; Sukanto, Boid and Jones, 1994).

Factors affecting DNA separation by pulsed-field gel electrophoresis

There are a number of factors that affect the efficiency of PFGE to separate DNA of different sizes; these include 1) Pulse time, 2) Voltage gradient, 3) Temperature, 4) Reorientation angle and 5) Agarose type and concentration.

Pulse times

The pulse time, also referred as switch interval or switch time, is defined as the length of time each alternating electric fields is activated for (Birren and Lai, 1993). Schwartz and Cantor, (1984) suggested that pulse times have a dramatic effect on DNA mobility with separation of bigger DNA requiring longer pulse times. The time required for DNA molecules to reorient in response to a change in electric field direction has been shown to be size dependent (Schwartz and Cantor, 1984; Birren, Lai, Clark *et al.*, 1988) with small molecules move faster than larger ones. Larger molecules require longer pulse time than small molecules for reorientation then migration in the gel. Megabase size molecules are sensitive to small changes in the pulse time, for example, small alteration between 25 min and 26.5 min or 30 min can cause a dramatic change in the migration of the megabase fragments of yeast chromosomal DNA (Birren and Lai, 1993).

Voltage gradient

A voltage gradient is the difference in electrical potential between a pair of electrodes and is the driving force that moves DNA through the agarose gel; it is measured in volts per centimetre. The voltage gradient can only be measured, as the voltage applied divided by the distance between the electrodes, for devices with homogeneous fields and reorientation angles. In PFGE, a voltage gradient of 6-10 V/cm is usually applied to resolve molecules up to 1 Mb, however, lower voltages are required for separating molecules larger than 2 Mb (Birren *et al.*, 1988). The DNA size range separated is affected by varying the voltage in PFGE, the same effect as when using different pulse times (Birren and Lai, 1993). This was shown by the DNA separation in several PFGE runs with variable voltages but a constant pulse time. Running the PFGE at lower voltage (for example, 3V/cm) separates smaller molecules (up to 400 kb); larger DNA molecules (1,500 kb) were separated when higher pulse time (6V/cm) was applied.

Temperature

In PFGE, buffer recirculation is essential to maintain a uniform temperature across the gel for the duration of the electrophoresis run. The temperature influences the DNA mobility in the agarose, DNA molecules move faster when the electrophoresis temperature is increased. Lai *et al.* (1989) showed that the migration of lambda DNA was twice as quick when run at 34^0 C compared with that at 4^0 C. Although DNA moves faster when the temperature is increased this generally yields poor resolution (Birren *et al.*, 1988). Gels run at low temperatures offer high resolution but at very low migration rates and the electrophoresis takes a very long time. Temperatures between 14^0 C and 22^0 C are generally regarded as

the best compromise between speed and best resolution (Lai *et al.*, 1989). Because DNA migrates at different rates at different temperatures, when the buffer temperature is changed, the switch interval must also be altered in order to reproduce a separation achieved at a different temperature. Lowering the buffer temperature requires switch intervals to be increased; increasing the temperature requires the switch intervals to be reduced to produce similar resolution for a specific DNA size range.

Running buffers

The resolution and mobility of DNA is affected by the composition of the electrophoresis buffer. The most commonly used buffers in PFGE are Tris-acetate and Tris-borate. Although DNA migrates faster in low molarity (ionic strength) buffers, they also have much lower buffering capacities which could lead to the buffer becoming exhausted during an electrophoresis run with the consequent failure of the gel to separate DNA fragments (Birren *et al.*, 1988). In early versions of the GeneLine PFGE systems, Beckman U.S.A. recommended using TAE buffers for highest resolution of DNA fragments. Fragments run faster in TAE buffers compared to the equivalent TBE buffers, but at the cost of changing the TAE buffer every 24-36 hours to maintain buffer integrity (Birren and Lai, 1993). Choice of buffer is therefore a compromise between run length and buffering capacity. TAE is useful for separating fragments >2Mb largely because of the faster migration of DNA in this buffer which allows a lower voltage to be used which itself prevents the buffer breaking down during electrophoresis. As with buffer temperature, switch intervals must be altered if one buffer type is substituted for another with 15% shorter switch intervals required when TAE is substituted for TBE.

Reorientation angle

The reorientation angle is the angle between the alternating electric fields, that is, the different directions that the DNA molecules migrate down the gel and affects the banding pattern resolution in PFGE (Schwartz and Cantor, 1984). Lai *et al.* (1989) showed that the separation of yeast chromosomal bands was almost identical when reorientation angles between 110^0 to 165^0 were used. It was also shown that a sharper banding pattern was achieved when the reorientation angle applied was 165^0, thus indicating that the sharpness of the bands is increased by wider reorientation angles.

However, it was shown (Lai *et al.*, 1989) that in the gels with progressively wider reorientation angles the DNA molecules will progressively slow down, which leads to sharper but more closely spaced bands than are obtained with uniform reorientation angles. This has shown that the width of the reorientation angles affects the DNA mobility in the gel. Since the velocity of the DNA migration reflects the sum of the electric field vectors, wider

reorientation angles will result in more time required for the DNA to move sideways in the gel and the less forward movement will be achieved. To achieve faster DNA separations, especially for molecules larger than 2 Mb. reorientation angles of approximately 106^0 are needed. For separating molecules smaller than 2 Mb. 120^0 reorientation angles are needed (Birren and Lai, 1993). In the TAFE system that has a fixed reorientation angle (115^0), good separation and the sharpness of the DNA fragments near the bottom of the gel are obtained by the changes of the electric field (Beckman, Bulletin No. 7863).

Type of agarose and agarose concentration

The separation of DNA molecules of different size depends predominantly on the sieving properties of the gel matrix. In PFGE both the type and concentration of the agarose influence the DNA separation and mobility. A wide variety of agaroses is available. The main properties for agarose used in electrophoresis are (1) the tensile strength of the gel, (2) the level of charged constituents present in the mixture, and (3) the degree of purity. The strength of the gel is important because it determines the ease of handling the gel. The amount of charge is measured in terms of electroendoosmosis (EEO). The DNA molecules move fastest in the low electroendoosmosis type agarose (Lai *et al.*, 1989). The presence of contaminants in the gel can degrade DNA. However, this can be avoided by using a molecular biology grade agarose, which has been assayed for the absence of contaminants such as proteases, nucleases and metal ions. Pulsed-field grade agarose is recommended for separating DNA larger than 2.5 Mb because it reduces the gel running time by up to 25% (Birren and Lai, 1993).

Alteration of the agarose concentration in PFGE affects the DNA mobility and the size range separated. Using a low concentration of agarose allows faster migration of the DNA molecules. Lai *et al.* (1989) showed that lambda DNA moves 50% faster in a 0.6% agarose than in a 1% agarose gel. However, higher agarose concentration can improve both the banding pattern resolution and band sharpness although only for a small range of DNA sizes (Birren *et al.*, 1988). It was shown that (Lai *et al.*, 1989) when the concentration of agarose is decreased the DNA migrates faster, a larger size range is separated and less sharp bands appeared. It was suggested to use 1% agarose for separating DNA fragments in the size range of 1 kb to 2.5 Mb and 0.7 or 0.8 % agarose for larger fragments. Wert and Furst (1988), however, stated that agarose concentration has minimal impact on PFGE and other gel concentrations may have similar performance with respect to pulse time and field strength.

2.4.2.2. Molecular karyotype

The development of PFGE has facilitated the separation of large, chromosome-sized DNA molecules. Applications of PFGE for studying the protozoan genome have led to the characterisation of some features of their karyotypes. Pulsed-field gel electrophoresis is a very useful tool for the study of chromosome number and sizes, karyotype polymorphism among different strains and the mechanisms behind the observed polymorphism (Henriksson, Aslund and Petterson, 1996a). In general karyotype patterns of trypanosomes can be divided into four size classes: 1) very large chromosomal DNA unresolved by PFGE which remain in or close to the gel slot (about 60% of the DNA, Van der Ploeg *et al.*, 1984a); 2) large chromosomal DNA of megabase size range located in the compression zone; 3) intermediate size chromosomes 200-1000 kb size range and, 4) mini chromosomes in the size range of 50-150 kb (Gottesdiener *et al.*, 1990; Van der Ploeg *et al.*, 1984a). The chromosomal size class allocations vary in different species and strains. In order to obtain "complete" karyotypes, several runs have to be carried out under different running conditions, each optimised to resolve a particular chromosomal class size.

2.4.2.3. Karyotype variability

Considerable differences in the karyotype patterns have been shown among different trypanosome species and strains. The karyotype of *T. evansi* has been determined in stocks originating from China (Lun *et al.*, 1992b), Kenya (Waitumbi, Murphy and Peregrine, 1994; Waitumbi and Young, 1994) and Indonesia (Boid *et al.*, 1992; Sukanto *et al.*, 1992; Sukanto *et al.*, 1994). The main features of *T. evansi* stocks from China, Kenya and Indonesia are presented in **Table 2.6**. The chromosomal DNA of *T. evansi* from China, (Lun *et al.*, 1992b) separated by a CHEF electrophoresis system, was composed of four chromosomal size classes: very large DNA which remain in the gel slot; about 2-3 bands located in the 1020-1125 kb size range; 4-5 bands between 290-1020 kb and a smear of mini-chromosomes with sizes smaller than 245 kb. Waitumbi, *et al.* (1994) also used the CHEF system to resolve bands between 50-600 kb size range and described the karyotype of *T. evansi* in three main chromosome size classes: chromosomes that remain in the gel slot; several discrete intermediate size chromosomal bands between 150-600 kb size range and a large number of minichromosomes between 50-150 kb size range. The minichromosomes were larger than the ones present in the control *T. brucei*. The karyotype study on the Indonesian stocks used a Beckman TAFE system to separate *T. evansi* chromosomal DNA. It was reported that the TAFE system resolved 16-20 chromosome bands of Indonesian *T. evansi* between 50-400 kb range (Boid *et al.*, 1992).

The main features of the molecular karyotypes of different species of trypanosomes and other members of Kinetoplastida, *Crithidia* and *Leishmania* are presented in **Table 2.6**.

The main difference between the karyotypes of *T. brucei* and *T. evansi* is the presence of mini-chromosomes. *Trypanosoma evansi* has few (3-20) mini-chromosomes whereas *T. brucei* contains about 100 mini-chromosomes. Some *T. brucei* stocks, however, contain only a few mini-chromosomes (Van der Ploeg *et al.*, 1984a; Van der Ploeg, Cornelissen, Michels, *et al.*, 1984b). The mini-chromosomes of *T. equiperdum* are reported to be larger and less heterogeneous in size than those of *T. evansi* (Lun *et al.*, 1992b). It was reported that *T. theileri* does not possess mini-chromosomes between 40-200 kb size range; and there are 17-18 chromosome bands between 400-2200 kb size range and the intermediate size chromosome numbers (between 400-900 kb size range) are variable (Bose, Petersen, Pospichal *et al.*, 1993). The mini-chromosomes are also absent in *T. rangeli*, the non-pathogenic human trypanosome (Henriksson, Solari, Rydaker, *et al.*, 1996b).

The mini-chromosomes (50-200 kb) although present in some *T. cruzi* stocks (Engman, Reddy, Donelson, *et al.*, 1987; Aymerich and Goldenberg, 1989) are not confirmed in all stocks (Gibson and Miles, 1986; Henriksson, Aslund, Macina *et al.*, 1990; Henriksson, Petterson and Solari, 1993; Henriksson, Porcel, Rydaker, *et al.*, 1995; Cano *et al.*, 1995). The absence of mini-chromosomes was reported in *T. vivax* (Gibson and Borst, 1986; Van der Ploeg *et al.*, 1984b), however, Dickin and Gibson (1989) detected a few of mini-chromosomes in *T. vivax*. The absence of mini-chromosomes were observed in *Crithidia* (Van der Ploeg *et al.*, 1984b) and *Leishmania* (Giannini, Schittini, Keithly, *et al.*, 1986; Pages, Bastien, Veas, *et al.*, 1989; Van der Ploeg *et al.*, 1984a).

Inter- and intra-strain heterogeneity was reported among different isolates of trypanosomes with regard to their chromosome number and size and the localisation of specific genes (Engman *et al.*, 1987). The karyotype pattern variability in trypanosomes might be caused by several factors. Variations in the DNA content, (Engman *et al.*, 1987) which varies up to 40% in *T. cruzi* (Dvorak *et al.*, 1982; Kooy *et al.*, 1989), had been implied to cause karyotype pattern variability. Other factors including telomeric growth (Bernards, Michels, Lincke *et al.*, 1983) and chromosomal rearrangements (Van der Ploeg *et al.*, 1984a; Shea, Glass, Parangi *et al.*, 1986) would alter the length of chromosomes in intermediate size range.

Table 2.6. Main features of the karyotypes of 12 kinetoplastid species

Species	Large chromosomes		Intermediate chromosome		Minichromosomes	
	Size (Mb)	Number	Size (kb)	Number	Size (kb)	Number
T. evansi:						
China [1]	1-1.1	2-3	290-1020	4-5	<245	Smear
Kenya [2]	>2	NS	150-600	5-7	50-150	Many
Indonesia [3,4]	>1	1-2	400-1000	2-5	50-250	3-7
					50-400	16-20
T. brucei [5,6]	1-2	3-6	200-700	>6	50-150	100 or few
T. equiperdum [7, 15]	~ 1	1	290-1020	5	<245	~10 or smear
T. theileri [8]	~2	1-2	400-900	NS		0
	1-1.8	9				
T. rangeli [9]	3-6	few	350-1500	16-20		0
T. congolense [10]	1-2	NS	150-450	NS	75-150	Smear
T. cruzi [11, 12, 13]	>2	NS	450-1600	20	50-150	Few or absent
T. vivax [14]	1->2	~10		0		0
C. fasciculata [15]	0.7-2	>10		0		0
L. major [16, 17]	1-3	Several	450-900	~10		0
	0.4-4	>17				
L. infantum [18]	1.5-2.6	7	300-1000	19		0
L. ctenocephali [14]	0.7-4	>20		0		0

NS: Chromosomal bands were not well separated, the exact number of chromosomes cannot be determined.

0: Chromosomal bands are not detected or absent.

1) Lun *et al.*, 1992b.
2) Waitumbi & Young, 1994.
3) Sukanto, Boid & Jones, 1994 (CTVM Annual Report 1993-1994. The University of Edinburgh. Pp 75-77).
4) Boid *et al.*, 1992.
5) Melville, 1998.
6) Van der Ploeg *et al.*, 1984a; b.
7) Lun *et al.*, 1992b.
8) Bose *et al.*, 1993.
9) Henriksson *et al.*, 1996b.
10) Masake *et al.*, 1988.
11) Engman *et al.*, 1987; Henriksson *et al.*, 1993.
12) Engman *et al.*, 1987; Aymerich & Goldenberg, 1989.
13) Gibson & Borst, 1986; Van der Ploeg *et al.*, 1984b; Majiwa *et al.*, 1985.
14) Dirie *et al.*, 1993a.
15) Van der Ploeg *et al.*, 1984a.
16) Giannini *et al.*, 1990.
17) Spithil and Samaras, 1985.
18) Pages *et al.* 1989.

2.4.2.3.1. Stability in the karyotype pattern

The karyotype patterns shown by PFGE are reproducible with regard to the number, sizes, and intensities of the chromosomal bands seen; 1) among different cryopreserved preparations of the same strain or clone; 2) after passage to different hosts, laboratory animals or culture; 3) over prolonged periods of cryopreservation; 4) over prolonged period between collections of stocks in the same area.

Karyotype stability in *T. evansi* has been observed in Kenya (Waitumbi and Young, 1994). The major chromosome profiles of *T. evansi* stocks in one area in Kenya remained stable after being collected 6 years apart (Waitumbi and Young, 1994). It was suggested that intervention of trypanocidal drugs had played an important role on the karyotype stability in *T. evansi* in Kenya (Waitumbi and Young, 1994). The limited heterogeneity in *T. evansi* chromosome profiles found in isolates from China might represent karyotype stability in that country (Lun *et al.*, 1992b). Karyotype stability has been shown in *T. congolense* after passage in mice, cattle, goats and tsetse for a period up to 12 months, indicating that deletion and translocation of whole chromosomes in *T. congolense* may not occur at such a high frequency as that observed in *T. brucei* (Masake *et al.*, 1988).

It has been suggested that karyotype analysis is a useful tool in establishing relationships among isolates since a high degree of similarity indicates a high degree of genetic homology (Giannini *et al.*, 1986).

2.4.2.3.2. Chromosome organisations

Chromosome organisation in trypanosomes separated by PFGE has been classified as mini, intermediate- and large chromosomes. The mini-chromosomes which are linear, aneuploid molecules contain telomeres, multiple copies of a 177 bp repeat and reservoir of non expressed VSG genes (Van der Ploeg, Smith, Polvere *et al.*, 1989; Weiden, Osheim, Beyer *et al.*, 1991). Although the main function of the mini-chromosomes is to provide a large pool of telomeric VSG genes, the presence of this chromosomes is not necessary for antigenic variation (Van der Ploeg *et al.*, 1984b). It has been shown that the presence of a large array of small chromosomes is not a general feature of trypanosomes that undergo antigenic variation: *T. equiperdum* has few mini-chromosomes (Van der Ploeg, Cornelissen, Barry, *et al.*, 1984c; Lun *et al.*, 1992b) and *T. vivax* has none (Van der Ploeg *et al.*, 1984b; Majiwa, Masake, Nantulya, *et al.*, 1985; Gibson and Borst, 1986) or few (Dickin and Gibson, 1989), both species undergo antigenic variation.

The intermediate chromosomes also differ in size and number between and within species. There are different numbers of intermediate sized chromosomes in the range 150 to 700 kb

within individual trypanosome species (Jasmer, Feagin, Payne, *et al.*, 1987). Size changes in the intermediate chromosomes also occur among cloned lines of *T. brucei* (Van der Ploeg *et al.*, 1984b; Shea *et al.*, 1986) as a result of duplicative transposition of VSG genes (Van der Ploeg *et al.*, 1984b). The intermediate chromosomes contain the 177 bp satellite sequence, VSG genes and a VSG expression site, but the remaining sequence content is uncharacterised (Swindle and Tait, 1996; Melville, 1998).

In addition to mini-and intermediate chromosomes, trypanosomes also have large chromosomes which contain housekeeping genes (Gibson *et al.*, 1985; Gottesdiener *et al.*, 1990), VSG genes (Van der Ploeg *et al.*, 1989) and VSG expression sites (Pays, Coquelet, Pays, *et al.*, 1989). Gottesdiener *et al.* (1990) determined the localisation of 18 genetic markers in large chromosomes (> 1 Mb) and showed evidence for both diploidy in large chromosomes and evidence that the homologous chromosomes could differ in size by several hundreds kilobase pairs.

Turner, Melville and Tait (1997) proposed a common system for identifying and naming chromosomes in *T. brucei*. Turner *et al.* (1997) used a *T. brucei* stock and separated the chromosomal DNA in the size range above the intermediate chromosomes (>1 Mb). A small set of highly conserved gene markers was then identified by Southern blot hybridisation of the PFGE separation of the stock. The data derived from the hybridisation of the specific markers had eight defined chromosomes in *T. brucei* in the size range between 1-3 Mb.

2.4.2.4. DNA probes

Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) based probes are available for parasite detection and characterisation. DNA probes were used initially for diagnostic purposes including diagnosis of parasitic diseases for which the causative organism was difficult to isolate and identify. Further research on DNA probes brought a wider utilisation of the probes for characterising parasites for epidemiological studies and disease control strategies.

Species specific probes have been used for the diagnosis of African trypanosomiasis (Massamba and Williams, 1984) and for detecting trypanosome species in tsetse flies (Kukla, Majiwa, Young, *et al.*, 1987). A VSG gene probe has been used to distinguish between *T. evansi* and *T. brucei* subspecies (Songa *et al.*, 1990) and within *T.b. gambiense* substrains (Barnes, Mottram, Selkirk *et al.*, 1989).

Masiga and Gibson (1990) used cloned kinetoplast DNA (kDNA) minicircle fragments as probes to distinguish *T. evansi* from other morphologically identical members of *Trypanozoon*. Two probes were produced: Probe A reacted only with the major isoenzyme group of *T. evansi* stocks from South America, Kenya, Sudan, Nigeria and Kuwait which

have type A minicircles; and probe B which reacted only with *T. evansi* stocks from Kenya which have type B minicircles and belonged to the minor isoenzyme group.

Hide, Cattand, LeRay *et al.* (1990) produced repetitive DNA sequences, which contain the ribosomal coding region (λ104) and one representing the ribosomal non-transcribed spacer region (λ109). The probes distinguished four basic groupings in subspecies of *T. brucei*: 1) *T.b. gambiense*; 2) East African stocks that infects both human (*T.b. rhodesiense*) and animals (*T.b. brucei*); 3) West African stocks that infect human and man, which was defined as *T.b. rhodesiense* and *T.b. brucei* respectively; 4) *T.b. rhodesiense* stocks from Zambia. Further studies showed that the trypanosome strains from Zambia had more distinct patterns than those from Kenya and Uganda, and confirmed that stocks from Zambia were *T.b. rhodesiense* (Hide, Buchanan, Welburn *et al.*, 1991). The probe corresponding to the non-transcribed spacer region (λ109) was also used to identify different species in the genus *Trypanozoon*: *T. evansi*, *T. equiperdum* and *T.b. brucei* (Zhang and Baltz, 1994). Zhang and Baltz (1994) used two probes: the λ109 probe (Hide *et al.*, 1990) and PBE2 probe that represents the *T. b. brucei* repetitive DNA sequences of unknown function (Jenni *et al.*, 1986; Hide *et al.*, 1990) which distinguished *T. evansi* stock patterns from China to that from Africa, South America and the Philippines. The application of the repetitive gene probes might be useful for distinguishing closely related trypanosomes in the epidemiological studies in trypanosomes isolated from widely distributed areas. However, this technique involves long steps of DNA separation by electrophoresis, Southern blotting and then hybridisation with specific gene probes.

2.4.2.5. Restriction Fragment Length Polymorphisms (RFLP)

Characterisation of different parasite strains can be determined by examining variation in the DNA sequence. This variation can be recognised using a series of restriction enzymes, which cut the DNA at specific sites. The polymorphisms resulting from the restriction enzyme digestion can be detected by the presence or absence of the particular enzyme site. The RFLP analysis involves several steps: restriction enzyme digestion of the parasite DNA, separation of the digested DNA by electrophoresis then Southern blot transfer of the separated DNA on to a membrane (Southern, 1975) followed by hybridisation with a labelled DNA probe and assessment of the variation in the banding patterns after hybridisation.

The probe used for the hybridisation can be a cloned fragment of any region of DNA, which shows polymorphisms between groups or individuals being examined (Hide and Tait, 1991). For example, single copy housekeeping genes (Gibson *et al.*, 1985); multicopy gene families including tubulin (Gibson *et al.*, 1985, Gibson and Borst, 1986; Cruz Tavares *et al.*, 1992), ribosomal genes (Hide *et al.*, 1990; Hide, *et al.*, 1991; Lun *et al.*, 1992b) or highly repetitive

DNA sequences (Gibson, Dukes and Gashumba, 1988; Dickin and Gibson, 1989; Hide, *et al.*, 1990; 1991; Zhang and Baltz, 1994).

Strain-specific complexity in the DNA banding patterns (fingerprints) was shown in the RFLP analysis using cloned antigen genes from *T. cruzi* (Henriksson *et al.*, 1990). Majiwa and Webster (1987) used repetitive DNA probe of *T. simiae*, which did not hybridise with other trypanosome species. Gibson *et al.* (1988) used probes that were species specific, each probe consist of repeat units of the major repetitive DNA of each species or intra-specific group. The repetitive probe of *T. brucei* only hybridised with every *Trypanozoon* species and three specific repetitive DNA probes of *T. congolense* specifically detected each type of *T. congolense* (Savannah, forest and Kenya coast).

DNA fingerprinting based on southern blotting with repetitive DNA probes followed by cluster analysis of the banding patterns has been used to determine the relationship between *T. brucei* stocks (Hide *et al.*, 1990). Further studies using the repetitive probes to identify *T. brucei* strains causing an outbreak of human sleeping sickness in Zambia showed them to have a distinct pattern from those collected in Kenya and Uganda (Hide *et al.*, 1990, 1991). These workers suggested that analysis of the RFLP patterns could provide a means to establish levels of identity and difference between trypanosome stocks based on variations in multiple loci.

The restriction fragment length polymorphisms (RLFP) have been applied to distinguish closely related trypanosome stocks, particularly in *Trypanozoon* where the relationships between different but morphologically similar trypanosomes are complex (Hide and Tait, 1991). Lun *et al.* (1992b) used VSG 118 and rDNA gene probes to hybridise *T. evansi* and *T. equiperdum* nDNA which had been digested with *Hind*III and *Pst*I; they found no differences among the Chinese stocks of *T. evansi* or *T. equiperdum* tested. The phylogenetic relatedness of *T. evansi* stocks collected from China, the Philippines, Africa and South America; *T. equiperdum* and *T. brucei* stocks was studied (Zhang and Baltz, 1994). The workers used southern blot analysis of restriction enzyme digested DNA, probed with two repetitive DNA sequences of *T. brucei*: λ109 (corresponds to the ribosomal non-transcribed spacer region) (Hide *et al.*, 1990) and pBE2, which corresponds to *T. brucei* repetitive DNA sequences of unknown function (Jenni *et al.*, 1986; Hide *et al.*, 1990). RFLP analysis revealed 3 groups of related stocks. Each probe showed that the 14 Chinese stocks were identical but differed from the African, South American and the Philippine stocks. The *T. brucei* stock was placed in a distinct group. While other subgroups differ, clear similarity showed that RFLP can be used to distinguish closely related trypanosomes (Zhang and Baltz, 1994). Other studies (Paindavoine *et al.*, 1986b) which applied RFLP have differentiated *T.b. gambiense* from *T.b. brucei* and *T.b. rhodesiense* using trypanosome surface antigen and undefined genomic DNA probes and showed that *T.b. gambiense* had a

more distinct pattern from other members of *Trypanozoon*: *T.b. brucei*, *T.b. rhodesiense* and *T. evansi.*

2.4.2.6. Polymerase Chain Reaction (PCR)

Mullis and Faloona (1987) devised a technique in which a nucleic acid sequence can be exponentially amplified *in vitro*. The technique, later known as the polymerase chain reaction (PCR), exploits polymerase, an enzyme that synthesizes DNA in the cell and the phenomenon of 'complementarity', where any two single-stranded DNA molecules with complementary sequences will bind together, under the right conditions, to form a double stranded DNA.

In PCR amplification, a source of double stranded DNA is mixed with the four deoxyribnucleoside triphosphates (dNTPs). A thermostable DNA polymerase is added to the mixture along with two oligonucleotide primers. These oligonucleotides are synthetic DNA strands, usually consisting of about 20 bases in length that are complementary to regions at either end of the target sequence. These oligonucleotides act as primers in the replication of the DNA. They anneal to opposite ends of the target DNA sequence and direct synthesis in opposite directions. The length of the DNA product is defined by the distance between the primers. In PCR therefore, detailed knowledge of the sequences at the ends of the target DNA fragment to be amplified is required.

The PCR was first applied in the prenatal diagnosis of sickle cell anaemia (Saiki, Arnheim and Erlich, 1985). The technique is fast, has great sensitivity and has the advantage that crude samples such as DNA contained in hair root or ancient specimens can readily be used as templates. The application of PCR has offered an alternative to techniques such as DNA sequencing, or to enhance the sensitivity of diagnosis that is not possible to carry out by conventional techniques.

The PCR has mainly been used for diagnostic purposes. In blood parasites, especially intracellular haemoparasites, diagnosis of the disease is often difficult where the level of parasitaemia is low and animals with carrier status often remain undetected. It was reported (Tanaka, Onoe, Matsuba *et al.*, 1993) that PCR amplification is sensitive and specific to detect *Theileria sergenti* infection in cattle. The PCR assay was applied to detect latent infections in carrier cattle. Figueroa, Chieves, Johnson *et al.* (1992) detected *Babesia bigemina* and Fahrimal, Goff and Jasmer (1992) detected *B. bovis* in infected erythrocytes in carrier cattle. Further, a multiplex PCR was developed using a combination of several sets of primer pairs to detect multiple infections in an animal. Figueroa, *et al.* (1992) applied the multiplex PCR using a combination of 3 primer sets specific for *Babesia bovis*, *B. bigemina*

and *Anaplasma marginale* show that the technique is also useful for detecting mixed infections in cattle as well as detecting latent infection in a single assay.

The PCR assay had been used for detecting the presence veterinary pathogen carried by ectoparasites, for example the detection of *Borrelia burgdorferi*, the agent of Lyme disease, in Ixodes ticks (Persing, 1991) and arthropod-borne virus transmitted by blood feeding arthropods (Ward, Marriott, Booth *et al.*, 1990). In trypanosomiasis, the PCR assay detected trypanosome infection in the saliva of the tsetse flies (Majiwa, Thatthi, Moloo *et al.*, 1994). The assay, which is a multiplex PCR, could also detect the presence of more than one trypanosome species in a fly. The presence of trypanosomal DNA in blood samples from animals that were not parasitaemic by microscopical examinations could also be detected with these primers.

Specific primers derived from minicircle sequences of *T. evansi* have been produced by Artama *et al.* (1992) who reported that a 373 bp PCR product was present in *T. evansi* but absent from *T. brucei*, *T. cruzi* and *Leishmania donovani*. Wuyts, Chokesajjawatee and Panyim (1994) used other primers constructed from repetitive sequence *T. evansi* DNA to detect *T. evansi* infection in cattle in Thailand. The test was sensitive, capable of detecting 0.5 pg of purified DNA per reaction, or the equivalent of 5 trypanosomes (assuming that one trypanosome has a DNA content of 0.1 pg) (Borst *et al.*, 1982). The test was specific for *T. evansi*, with no reaction with *Theileria*, *Anaplasma*, *Babesia* and *Plasmodium* (Wuyts, Chokesajjawatee, Sarataphan, *et al.*, 1995). However, these primers could not be used to differentiate between *T. brucei* and *T. equiperdum* as they generated the same amplification products (Wuyts *et al.*, 1994). Overall, the PCR has provided a specific and sensitive diagnostic tool for the identification of trypanosome infection, which could be useful in the epidemiological studies.

2.4.2.7. Random amplified polymorphic DNA (RAPD) analysis

A polymerase chain reaction technique was developed based on the amplification of short single oligonucleotide primers of arbitrary sequence of the genomic DNA being studied (Williams, Kubelik, Livak *et al.*, 1990; Welsh and McClelland, 1990). This technique, called random amplified polymorphic DNA (RAPD), uses primers of 10 to 12 mers in length instead of the pairs of longer oligonucleotides used as primers in PCR.

Other modifications of RAPD analysis have been described as DNA amplification fingerprinting (DAF) and arbitrarily primed PCR (AP-PCR). The DNA amplification fingerprinting (DAF) developed by Caetano-Anolles, Bassam and Gresshoff (1991) uses very short random primers (5-8 bases) and generates greater numbers of fragments than RAPD analysis. The banding patterns after DAF analysis is visualised by polyacrylamide gel

electrophoresis and silver staining (Caetano-Anolles *et al.*, 1991). Although DAF generates complex products, many of them are polymorphic resulting in characteristic DNA fingerprints (Caetano-Anolles *et al.*, 1991). The arbitrarily primed PCR (AP-PCR) described by Welsh and McClelland (1990) uses single longer primers (20-30 bases). The amplification products after AP-PCR are radioactively labelled, resolved by polyacrylamide gel electrophoresis and then visualised by autoradiography.

The principal advantage of the RAPD technique is that single short oligonucleotide primers are sufficient to differentiate between organisms and genomic fingerprints can be obtained without the need for any prior DNA sequence knowledge (Williams *et al.*, 1990; Welsh and McClelland, 1990). The random amplified polymorphic DNA technique has provided a relatively simple way to analyse polymorphic DNA markers from a wide variety of eukaryote and prokaryote species. Fingerprinting of 15 stocks of *Candida albicans* by RAPD analysis detected 11 patterns compared to 14 patterns detected by PFGE separation, a level of discrimination approaching that of PFGE (Bostock, Khattak, Matthews, *et al.*, 1993).

The RAPD analysis detects genetic variation at a finer level than that by isoenzyme electrophoresis (Stevens and Tibayrenc, 1995; Steindel, Neto, Pinto, *et al.*, 1994). The RAPD analysis has some advantages over enzyme electrophoresis particularly in the larger numbers of loci detected by RAPD analysis and using much less material (Steindel *et al.*, 1994). Approximately 10^3 parasites are sufficient for 10 reactions in RAPD analysis compared to 10^8 parasites required for enzyme electrophoresis (Steindel *et al.*, 1994). The disadvantage of the RAPD analysis is that there should be no contamination with host DNA because it affects the fingerprint pattern (Waitumbi and Murphy, 1993; Bishop, Sohanpal, Morzaria, 1993).

The RAPD analysis had been extensively used for a number of reasons. Tibayrenc, Neubauer, Barnabe *et al.* (1993) suggested that the RAPD analysis is suitable for investigating genetic and evolutionary problems. The RAPD analysis has a good parity with results from multilocus enzyme electrophoresis confirming that RAPD markers are reliable genetic markers (Tibayrenc *et al.*, 1993). Steindel, Neto, de Menezes *et al.* (1993) suggested that the RAPD profiles of *T. cruzi* strains in the same zymodeme tended to be similar although not identical; the results also suggested the presence of a large number of individual genotypes within the species.

According to Hadrys, Balick and Schierwater (1992) RAPD analysis can be applied for: determination of taxonomic identity; analyses of interspecific geneflow and hybrid speciation; determination in paternity and kinship relationships; analysis of mixed genome samples and generation of novel specific probes. In the taxonomic study, the RAPD analysis is based on the determination of polymorphic and non-polymorphic patterns detected in the amplification products. These definitions are relative for a given operational taxonomic unit (OTU), that is,

the RAPD analysis of several individuals within a species or several species within a given genus (Hadrys *et al.*, 1992). It was also suggested that constant RAPD product fragments can be used for species or genus identification, and the polymorphic fragments detected at the species level can be used to identify members of a given species.

Both strains and species polymorphism has been detected by RAPD analysis in *Schistosoma* (Neto, de Souza, Rollinson *et al.*, 1993). Neto *et al.* (1993), however, stated that the degree of variation of the RAPD bands of *Schistosoma* species reveal more differences when species, rather than strains, are compared. Intra species differentiation in the RAPD profiles was detected in *Candida albicans* (Bostock, *et al.*, 1993), *T. cruzi* (Steindel, *et al.*, 1993), *Babesia bovis* (Lew, Dalrymple, Jeston *et al.*, 1997), *Trypanosoma rangeli* (Steindel, *et al.*, 1994), *Lucilia cuprina* (Stevens and Wall, 1997).

The RAPD technique has been used to characterise a number of trypanosome stocks. Waitumbi and Murphy (1993) used ILO525 primer and observed that the RAPD patterns shown by *T.b. brucei* were indistinguishable from those of *T.b. rhodesiense*. However, *T.b. gambiense* showed different patterns from those of *T.b. brucei* and *T.b. rhodesiense*. This finding was also supported by Kanmogne, Stevens, Asonganyi *et al.* (1996) who observed marked polymorphisms in the RAPD marker between *T.b. gambiense* and *T.b.* non-*gambiense* stocks. Intra-specific homogeneity in the RAPD pattern was, however, observed among *T. evansi* stocks from Kenya, (Waitumbi and Murphy, 1993; Waitumbi, *et al.*, 1994) but the pattern shown by *T. evansi* was different from that of *T. brucei* (Waitumbi and Murphy, 1993). Using the same primer, Waitumbi and Murphy (1993) showed intra-species heterogeneity in the RAPD patterns among *T. congolense* stocks, which could be related to the geographical region from where the isolates were collected: Savannah, Kilifi and West Africa types.

Stevens and Tibayrenc (1995) demonstrated linkage disequilibrium (LD) by RAPD analysis with *T. brucei* stocks isolated from tsetse flies. These workers suggested that the presence of such LD indicated that the trypanosome population circulating in tsetse flies may not be undergoing random mating as in the case of sexual reproduction. Studies carried out by Gibson (1990) and Cibulkis (1992), however, disagreed with this conclusion.

2.4.2.7.1. Mechanism of RAPD analysis

Despite the widespread application of RAPD analysis, few studies have been carried out to explore the molecular mechanisms of the RAPD phenomenon. Using mouse genomic DNA, Venugopal, Mohapatra and Salo (1993) studied the mechanism involved in the RAPD process. The low stringency annealing temperature in RAPD analysis (40^0 C) is designed so that the primers allow anneal to many binding sites, which then leads to many mismatches

(Welsh and McClelland, 1990). The RAPD banding pattern is generated by the amplification of these sequences between the regions where the primers bind to sites on both DNA strands (Welsh and McClelland, 1990). Two genomes with identical RAPD primer binding sites should generate identical RAPD products and any extra RAPD bands are only produced when the other genome contains extra binding sites (See **Figure 2.4**). It has to be emphasised however, that polymorphisms in the RAPD analysis can be generated by amplification resulting from imperfect pairing or multiple mismatching between primer and the DNA template (Williams *et al.*, 1990). Variations in the RAPD products may also be due to the deletions of a priming site or by insertions that cause priming sites too distant to support amplification (Williams *et al.*, 1990). At least one perfect primer annealing site is necessary for a given target site to be amplified in RAPD process.

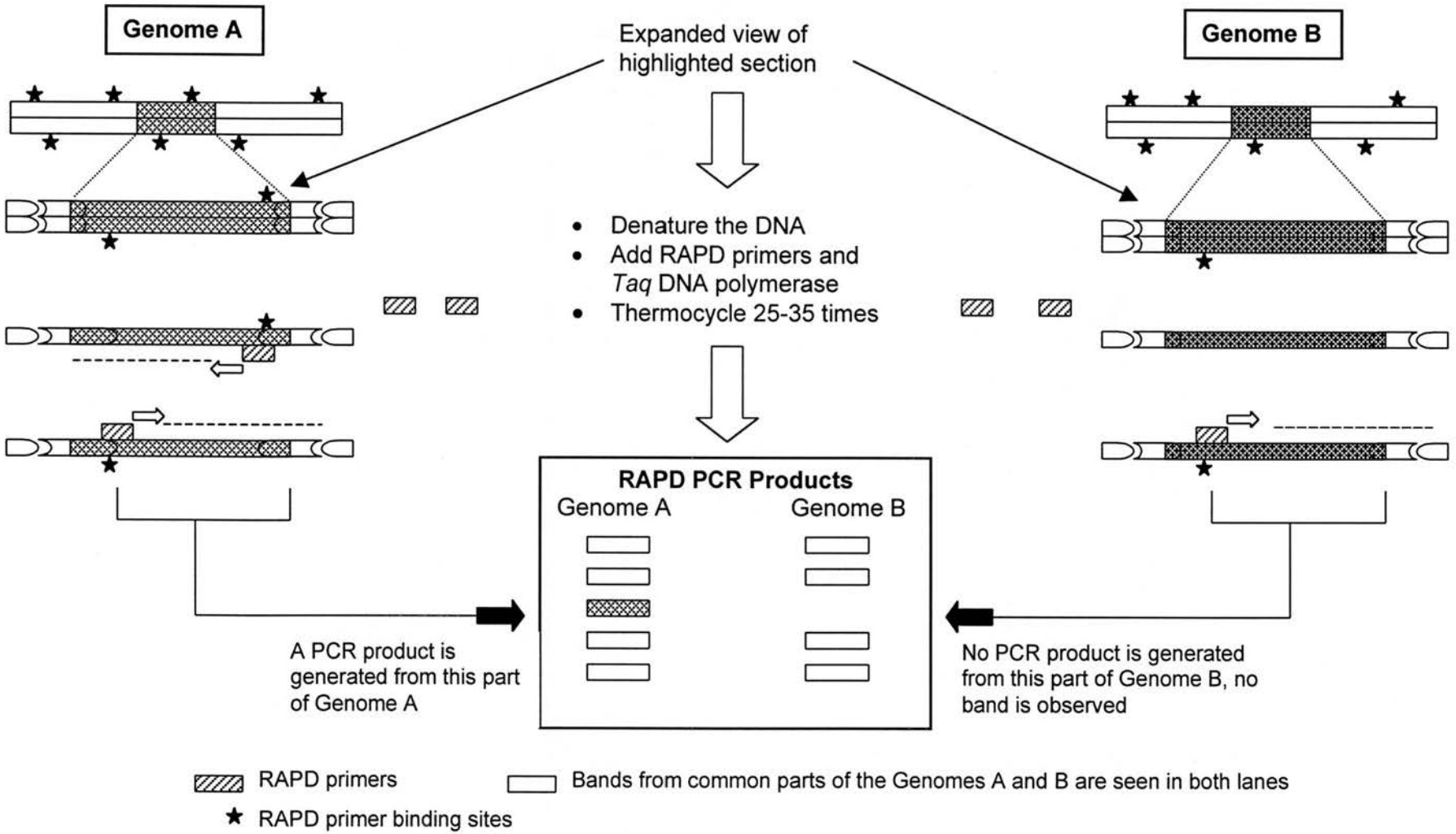
Venugopal *et al.* (1993) suggested that a single band generated by RAPD analysis does not necessarily mean single gene copy. A single RAPD band was excised, reamplified with the same primer and labelled. The labelled band was used to probe the mouse genomic DNA by Southern blot hybridisation. It was observed that the probe hybridised to two alleles in the genome indicating that the band did not represent single gene copies. This was confirmed by sequencing clones from a single RAPD band, which produced different sequences (Venugopal *et al.*, 1993).

Some of the RAPD bands can be generated by self-priming (Caetano-Anolles, 1993) due to the presence of palindromic "sticky" ends on primers, which can result in amplification by formation of hairpin loops. Self priming usually occurs at the 3' end of the first amplified strands, which then leads to the generation of large DNA strands in the RAPD products (Venugopal *et al.*, 1993). Furthermore it was suggested (Caetano-Anolles *et al.*, 1991) that the different intensities of the RAPD bands can be correlated with the number of copies of the amplification regions and/or the efficiency of the amplification in that region.

2.4.2.7.2. Factors affecting the reproducibility in RAPD analysis

A number of studies have been carried out to determine the reliability and reproducibility of RAPD analysis. Factors affecting conventional PCR such as Mg^{++} concentrations, primers concentration, enzyme source and concentration, DNA extraction methods, DNA concentration, the model of thermocycler used and laboratory practice also influence the fidelity of RAPD analysis (Tyler, Wang, Tyler, *et al.*, 1997).

Figure 2.4. RAPD banding pattern differences between two genomes



Effect of magnesium concentration

The Mg^{++} concentration contained in the RAPD reaction mixture was reported (Park and Kohel, 1994; Ellsworth, Rittenhouse and Honeycutt, 1993) to influence the RAPD products. Park and Kohel, (1994) suggested that optimum $MgCl_2$ concentration should be determined for each random primer to obtain consistent RAPD banding pattern and maximum polymorphism. They also reported that significant quantitative difference in RAPD bands and qualitative differences in RAPD banding pattern could be obtained by varying the $MgCl_2$ concentration in the reaction mixture. Ellsworth *et al.* (1993) obtained drastic alterations in RAPD profiles by varying the magnesium concentrations. At $MgCl_2$ concentration between 0 to 2 mM the appearance of the RAPD bands are not consistent and artifactual bands are present. Magnesium concentrations above 2 mM do not seem to influence the RAPD banding pattern (Ellsworth *et al.*, 1993).

Effects of DNA concentration and preparation

The quality of the DNA template is considered to be one of the principal factors influencing the fidelity of RAPD analysis. It has been reported that ethanol precipitable contaminants in DNA preparation are a major cause of irreproducibility in RAPD patterns (Micheli, Bova, Pascale *et al.*, 1994). It was also reported that the whole cell preparations used for DNA template affected the reproducibility of the RAPD analysis and are not recommended (Tyler *et al.*, 1997), possibly due to the presence of nucleases in the whole cell preparations causing degeneration of the DNA template. Welsh and McClelland, (1990) suggested that a DNA template prepared by proteinase K digestion followed by phenol extraction gives similar results in RAPD analysis to those obtained from the supernatant after centrifugation of boiled DNA samples.

The DNA template for RAPD analysis can be prepared from DNA in either TE (10 mM Tris, 1 mM EDTA) buffer or water and from DNA embedded in agarose. The effect of different DNA preparation samples on the consistent result of the RAPD analysis has been studied in relation to variations in Mg^{++} concentration (Schierwater and Enders, 1993). The results suggested that the RAPD pattern of the DNA resuspended in TE or water is more sensitive to slight changes in Mg^{++} concentration than that of the agarose embedded DNA, which generates more consistent results.

The DNA concentration can affect the RAPD pattern reproducibility as well as the quality. The optimal DNA concentration to obtain a stable pattern in RAPD analysis differs by different species. For example, for *Staphylococcus aureus* between 50 to 500 ng DNA is optimal (Tyler *et al.*, 1997), while Welsh and McClelland (1990) reported consistent results

were obtained at the DNA concentration of 30 pg to 7.5 ng for *S. aureus*. Other workers showed that DNA concentration of less than 10 ng showed the greatest variability among the RAPD results with 100 ng DNA produced the most consistent results (MacPherson, Eckstein, Scoles *et al.*, 1993). In the case of *Trypanosoma brucei,* 5-15 ng DNA resulted in reproducible RAPD patterns, however, DNA concentrations above this value (40 ng) result in reduced amplification products (Kanmogne *et al.*, 1996).

Other parameters

While all thermal cyclers are capable of generating reliable and reproducible RAPD patterns, different machines can produce different RAPD products (MacPherson *et al.*, 1993). The RAPD product variation was correlated to the temperature differences inside the tube between individual thermocycler (He, Viljanen and Mertsola, 1994). Day to day variations in the RAPD results was also reported to occur in two different thermocycler brands when used with a single primer (Meunier and Grimont, 1993).

Penner, Bush, Wise *et al.* (1993) observed variation in the RAPD products on two oat cultivars, which RAPD analysis were conducted in different laboratories. It was suggested (Penner *et al.*, 1993) that the lab to lab variation occurs due to the different protocols employed and the type of thermocycler used, which was recognised as the main source of variation in size range. Furthermore, Penner *et al.* (1993) suggested that reproducible RAPD pattern among laboratories can only be obtained when the overall temperature profiles, especially annealing temperature inside the tubes are identical. The RAPD analysis is more sensitive to the temperature difference than the conventional PCR due to the less stringent annealing temperatures and shorter primers used in RAPD analysis (He *et al.*, 1994).

The number of reaction cycles and the quantity of DNA are among critical factors in success of RAPD analysis (Neto, *et al.*, 1993). Very high cycle numbers were found to frequently result in amplification of non-specific bands in no-DNA control tubes, although the bands were not apparent in the presence of DNA (Neto *et al.*, 1993). The effect of an excessive number of reaction cycles was also reported by Bell and DeMarini (1991) who suggested that the non-specific bands are random-length high-molecular weight fragments generated from extension and random termination of annealing events of the 3'-OH ends of the amplified product to genomic template or to each other after most of the primer has been converted into PCR product (Bell and DeMarini, 1991).

Despite initial reports supporting the broad application of RAPD analysis, Tyler *et al.* (1997) suggested that the RAPD analysis has limited application and does not appear to be well-suited to defining the evolution of genetic relationships between organisms, tracking

epidemiological relatedness between species or surveying genetic variation in natural populations, due to its inability to discriminate between artifactual variation and true polymorphism.

The RAPD analysis was suggested (Bishop *et al.*, 1993) to have limitations for epidemiological applications due to the lack of consistency of the overall fingerprint, which was thought to be due to the variation in primer annealing between replicate RAPD reactions. This may be due to the complex reactions in which a single primer competes for priming at many sites in the genome. Further, it was observed (Bishop *et al.*, 1993) that inconsistent products are most evident in uncloned populations, as component of the clones may vary in sequence at priming sites.

Furthermore Tyler *et al.* (1997) had suggested that the majority of reproducibility problems were found with prokaryotic DNA. The RAPD products are more stable in eukaryotic DNA due to the larger genome size (Tyler *et al.*, 1997), which provides more binding sites and greater stability (Caetano-Anolles *et al.*, 1991). The RAPD analysis might be more suitable for large genomes (Tyler *et al.*, 1997) and the result of the analysis are reproducible when a standard set of primers and standard protocols could be established (Penner *et al.*, 1993). Phylogenetic classification for eukaryotic genome on the basis of band sharing among related species in the RAPD pattern analysis had been found useful. However, it should be noted that homologous bands resolved by electrophoresis do not necessarily represent the same region of amplified DNA (Petrie, Burg III and Cain, 1996).

2.4.2.8. Analysis using ribosomal RNA gene sequences

Most of the cellular RNA (80-85%) consists of ribosomal RNA, which has important functions in all organisms. The abundance of ribosomal RNA in organisms is suitable for sensitive parasite detection (Waters and McCutchan, 1989; Uliana, Affonso, Camargo *et al.*, 1991). The sensitivity can be increased by amplifying the ribosomal RNA genes or the RNA by polymerase chain reaction (Uliana *et al.*, 1991; Van Eys, Schoone, Kroon *et al.*, 1992).

The ribosomal RNA in the *Trypanosomatidae* comprises of large subunit (LSU) and small subunit (SSU) ribosomal RNA. Ribosomal RNA sequences have been used as a tool for defining the phylogenetic relationships among Trypanosomatidae (Lake, Cruz, Ferreira *et al.*, 1988; Hernandez, Rios, Valdes *et al.*, 1990; Gomez, Valdes, Pinero *et al.*, 1991) and distinguishing between related species, including members of this family (Waters and McCutchan, 1989; DeLong, Wickham and Pace, 1989; Amann, Springer, Ludwig *et al.*, 1991; Uliana *et al.*, 1991; Zarda, Amann, Wallner *et al.*, 1991).

2.4.2.8.1. Ribosomal RNA gene sequencing, cloning and probing

Determination of rRNA gene sequences is a technique for characterising differences between and within species of an organism. This involves sequencing of the gene, cloning of the specified gene and labeling to produce a probe. A unique nucleotide pattern had been found by analysing partial sequences of LSU and SSU rRNA, which detects inter-species differences in yeast (Kurtzman and Robnett, 1991). A comparison of the rRNA gene sequences (Sogin, Elwood and Gunderson, 1986a) of *Euglena gracilis* with those of *T. brucei* has shown a very distant relationship and a long separate evolutionary history between these two species. Benadives, Sullivan, Steuer *et al.* (1993) screened a number of oligonucleotide probes derived from LSU (24S or 28S) ribosomal RNA sequences of *T. cruzi* (Vierra de Arruda, Reinach, Colli *et al.*, 1990) and *Crithidia fasciculata* (Spencer, Collings, Schnare *et al.*, 1987) and found differences between *T. cruzi*, *T. rangeli* and *Leishmania*.

The small subunit rRNA sequences have been used in phylogenetic studies (Lane, Pace, Olsen *et al.*, 1985) because they have a high taxonomic information content, conservative nature and universal distribution. The SSU rRNA gene sequence of *L. donovani* has been investigated (Looker, Miller, Elwood *et al.*, 1988) and suggests that *L. donovani* is more closely related to *C. fasciculata* (97%) than to *T. brucei* (84%). Lane *et al.* (1985) developed a method to produce large blocks of SSU-rRNA (16S rRNA) sequence data rapidly without isolation or cloning of the SSU-rRNA gene. The purified RNA preparation was selectively targeted for dideoxynucleotide-terminated sequencing by reverse transcriptase and synthetic oligodeoxynucleotide primers that were complementary to the universally conserved SSU-rRNA sequences (Lane *et al.*, 1985).

A phylogenetic tree derived from the rRNA gene sequences was produced and suggesting that *C. fasciculata*, *T. brucei* and *T. cruzi* belonged to a different branch and put *C. fasciculata* and *T. cruzi* in a separate cluster from *T. brucei* (Gomez, *et al.*, 1991). Whether this reflects on evolutionary relationships is unknown, since to date, the definition of a genus among trypanosomatids is based on a combination of morphological stage and host range (Gomez *et al.*, 1991).

The analysis of rDNA fragment by cloning, sequencing and probing has become a well-developed technique in the study of taxonomy of bacteria, protozoa and fungi. With the development of PCR technique, the long step for cloning has been replaced by amplification of the required fragment sequence to provide sufficient rDNA material for sequencing. This technique, however, is still labour intensive.

Probes derived from amplified rRNA sequence were produced and used as a diagnostic tool for strain identification for *T. cruzi* (Souto and Zingales, 1993). The probes were produced from a sequence of approximately 100 bp of the 24Sα rRNA amplified by reverse

transcriptase-polymerase chain reaction (RT-PCR) followed by labelling. The probe was specific for *T. cruzi*, and there was no cross-reaction with *Leishmania* and *T. rangeli* (Souto and Zingales, 1993). Pelle (1993) described a quick technique to differentiate *T. brucei* from *T. congolense* by examining the banding patterns of the electrophoretic profile of the total rRNA. The technique, however, failed to distinguish between *T. brucei* and *T. vivax* (Pelle, 1993).

2.4.2.8.2. Ribotyping

Ribotyping, was developed by Stull, LiPuma and Edlind (1988) to detect polymorphism in rDNA sequences in bacteria. The method used restriction enzyme to digest genomic DNA and size fractionation of the digestion products on agarose gels. The gel was then Southern blotted on to a nylon membrane and reacted with a radioactive labelled rDNA. Ribotyping was applied in eukaryotes by Clark, Cross and DeJonckhere (1989) to assess inter- and intra-specific diversity and phylogenetic relationships in the genus *Naegleria*. Little variation was found in the pathogenic species of *N. fowleri* despite its worldwide distribution. It was shown to be closely related to the non-pathogenic *N. lovaniensis* (Clark *et al.*, 1989). Fernandes, Nelson and Beverley (1993) cloned and sequenced the whole PCR amplified 2.2 kb SSU rRNA gene from *Bodo caudatus*, *T. cruzi*, *Endotrypanum monterogei*, *Leptomonas* and *Leishmania donovani* and reported inter-species variation in the sequences among PCR-generated of the SSU rRNA gene. Uliana *et al.* (1991) detected inter-genus differences in *Leishmania* spp and *Trypanosoma* spp genomic DNA.

2.4.2.8.3. Riboprinting

Riboprinting is a PCR-based technique for fingerprinting eukaryotic ribosomal genes (Clark and Diamond, 1991a). This method is also known as ARDRA (amplified ribosomal DNA restriction analysis) as described by Vaneechoutte, Rossau, DeVos, *et al.* (1992) for rapid identification of bacteria.

In riboprinting, the extracted DNA is used as the template in PCR and the oligonucleotide primers used for the PCR amplification are generated from a highly conserved sequence of the ribosomal RNA gene located at the very 5' and 3' end of the SSU-rRNA gene (Clark and Diamond 1991a). Although the SSU-rDNA is highly conserved, there is sequence divergence in the region of the primer binding sites that do not amplify (Clark, 1997a). A large amount of complete sequence of SSU rDNA is amplified after the PCR is completed. It was observed (Clark, 1997a) that most of the eukaryotes amplified with SSU rDNA yield a fragment between 1,700-2,200 bp length. At this level species identification is impossible but when the amplified ribosomal DNA is digested with the restriction enzymes and the

fragments were size separated in agarose gel followed by ethidium bromide staining, banding pattern polymorphism can be detected. Riboprint pattern differences are generated by the restriction sites that fall in conserved and variable regions of the gene. In riboprinting, the long steps of transferring the DNA to be studied on a nylon membrane followed by hybridisation with the labelled probe are not needed.

Data generated from riboprinting has been used to construct a phylogenetic tree *Entamoeba* sp. (Clark and Diamond, 1997), *Crithidia* sp (Clark, 1997a), *Vahlkampfia* spp. (Brown and Jonckheere, 1994), *Saccharomyces* (Messner and Prillinger, 1995) and for diagnostic purposes for species identification of bacteria (Vaneechoutte *et al.*, 1992), *Toxoplasma gondii* (Brindley, Gazzinelli, Denkers *et al.*, 1993) and *Entamoeba gingivalis* (Clark and Diamond, 1992).

Application of riboprinting for inter- and intraspecies differentiation

Riboprint pattern differences have been used to distinguish between pathogenic and non-pathogenic strains of *E. histolytica* (Clark and Diamond, 1991b), later re-described as two different species, the pathogen *E. histolytica* and the non-pathogen *E. dispar* (Diamond and Clark, 1993). The existence of the two species had been confirmed by the differences (1.7%) between the 2 SSU rRNA gene sequences (Novati, Sironi, Granata *et al.*, 1996) and of other gene sequences (Tannich, Horstmann, Knobloch *et al.*, 1989; Tannich, Scholze, Nickel *et al.*, 1991; Bruchhaus, Jacobs, Leippe *et al.*, 1996). This has shown that riboprinting is a powerful tool with the potential to recognise the existence of two morphologically similar species (Clark, 1997a). The use of riboprinting as a diagnostic tool was shown in the detection of *Entamoeba gingivalis* in the uterus (Clark and Diamond, 1992). In coccidia, riboprinting was used to identify *Toxoplasma gondii* from morphologically indistinguishable proliferative stages of other zoonotic coccidia such as *Neospora caninum* and *Sarcocystis* sp, which can invade the same hosts. Clark, Martin and Diamond (1995) used riboprinting to detect inter-specific differences in trypanosomes isolated from toads and frogs, however, there were no intra-specific variations detected.

Intra-species variations in riboprint patterns were detected in *Entamoeba coli*, *E. gingivalis* and *E. moshkovskii* (Clark and Diamond, 1997). The intra-specific variations were then led to the groupings of the organisms into ribodemes for the population that sharing identical riboprint pattern (Clark and Diamond, 1997). Intra-specific riboprint variations was also observed in *T. cruzi* (Clark and Pung, 1994) and in *Blastocytis hominis*, a human intestinal parasite of uncertain role in human disease (Clark, 1997b). In yeast, heterogeneity in riboprint patterns was observed in 17 strains of *Saccharomyces cerevisiae* (Molina, Inoue and Jong, 1992). Riboprint data revealed 2 major groups, *S. cerevisiae* and *S. bayanus*, suggesting that *S. bayanus* should be reinstated as a separate taxon (Molina *et al.*, 1992).

The data generated by riboprinting had shown a remarkable correlation between the host species classification and riboprint group as reported in *T. cruzi* (Clark and Pung, 1994) and *Crithidia* sp (Clark, 1997a). Riboprint patterns of *T. cruzi* stocks isolated from opossum was different from those isolated from raccoons (Clark and Pung, 1994). In *Crithidia*, the riboprint pattern 3, which consisted of *C. fasciculata* stocks was isolated from insects of the family Culicidae and riboprint pattern 7, which consisted of several species of *Crithidia,* was isolated from the family Rudiividae (Clark, 1997a).

The riboprint data determined in *Crithidia* was then used to construct a phylogenetic tree among *Crithidia* species and showed distant relationships between riboprint pattern 1 (consisting of *Crithidia deanei*) and 2 (*C. oncopelti*) to the rest of *Crithidia* sp belonging to pattern 3-7 (Clark, 1997a). Phylogenetic trees based on riboprint data were also built to determine the taxonomic relationships between *Kluyveromyces* sp. (Shen, Jong and Molina, 1994) and *Vahlkampfia*, a free-living amoebae (Brown and DeJonckheere, 1994) showing that closely related species belonged to the same cluster.

2.5. AIMS

Trypanosoma evansi is endemic in livestock in Indonesia; however, outbreaks of surra are sporadic and often limited to particular areas of Indonesia. Differences in the virulence of strains of *T. evansi* from different areas have been observed on some occasions, which may be related to the differences in antigenicity of the strains involved in the outbreak. Such differences might be detectable at the molecular level, therefore, molecular-based techniques including PFGE, RAPD, Riboprinting and the simple sequence repeat-PCR (SSR-PCR) were used to examine the diversity of *T. evansi* stocks isolated from widely distributed areas of Indonesia in the hope that any differences seen might be related to geographical differences that could be used to explain local differences in virulence. The PFGE has the potential to distinguish individual populations based on the differences in chromosome size profiles. Karyotype analysis using TAFE was carried out to determine the distribution of molecular karyotype patterns and to investigate the significance of any karyotype differences with regard to their locations from where the stocks were isolated, their hosts from which they were derived, their differences associated with pathogenicity, changes in the surface variant antigenic types and their susceptibility to trypanocidal drug. The technique was also applied to study the chromosomal organisation and chromosomal localisation of six genetic markers (Phospholipase C, Cysteine Proteinase, *T. brucei* ribosomal RNA coding region, *T. brucei* tubulin, Aldolase and Glucose-6- phosphate isomerase).

RAPD analysis is a PCR-based technique that uses single primers of 10 to 12 mers in length instead of the pairs of longer oligonucleotides used in targeted PCR amplification (Williams *et al.*, 1990; Welsh and McClelland, 1990). RAPD analysis using the primer ILO525, which was used to demonstrate inter- and intra-species differences in *T. brucei* and *T. evansi* stocks from Kenya (Waitumbi and Murphy, 1993), was applied in the present study to examine differences among *T. evansi* stocks from Indonesia. RAPD analysis using other commercially available primers was also investigated to find suitable primer(s) that might be capable of detecting differences among the Indonesian stocks. A primer that could potentiality detect inter-stock differences was chosen from a pilot study and then used to test the stability of the RAPD profiles in *T. evansi* relapse populations and to examine the RAPD pattern distribution in the Indonesian stocks. The possibility of using DNA samples prepared directly from cryopreserved stabilates was investigated in order to maximise the application of RAPD analysis for epidemiological studies on a greater number of stocks from widely distributed areas.

Riboprinting detects variation within the conserved SSU rDNA sequences. Riboprinting involves PCR amplification of the SSU rRNA followed by restriction fragment length polymorphism with a range of restriction enzymes. Although the regions used for the primer are conserved, there are also variable regions that can be analysed by changes in restriction enzyme profiles. The aim of this study is to apply the riboprinting technique (Clark and Diamond, 1991a; Clark *et al.*, 1995) for detecting stock differences among *T. evansi* isolates from Indonesia.

The simple sequence repeat anchored PCR (SSR-PCR) uses a simple $(CA)_n$ repeat as a primer to amplify trypanosomal DNA. The present study was carried out to explore the possibility of using different genetic markers for *T. evansi* stock characterisation. The SSR-PCR used by Oliveira, Macedo, Chiari, *et al.* (1997) for studying genetic variability in *T. cruzi*, *Leishmania* and *Schistosoma* was adapted for use with *T. evansi* stocks from Indonesia.

Overall, therefore, the use of a range of analytical techniques for the examination of *T. evansi* characteristics at the DNA level was expected to detect sufficient inter-stock genetic variation that could be applied to a better understanding of the epidemiological of the parasite.

CHAPTER THREE

GENERAL MATERIALS AND METHODS

3.1. *TRYPANOSOMA EVANSI* STOCKS

Eighty *T. evansi* stocks were collected from naturally infected cattle, buffaloes and a horse in ten different regions in Indonesia which are known as endemic areas (Adiwinata and Dachlan, 1969; Payne *et al.*, 1991a). The type of livestock available in the areas sampled differed depending on the animal usage in each area. The animals are kept by smallholder farmers (each farmer has 2-3 animals) and most of the animals are housed in their stalls at night and grazed during the day, some are fed in their stalls (zero grazed). Extensive grazing and non-housed animals are preferred by local farmers in areas outside Java. However, in areas where transmigrants from Java and Bali are present, an intensive-farming system is practiced.

Trypanosoma evansi stocks were collected from cattle, buffaloes and horses with natural infection by three different methods:

- stocks that were collected once (32 stocks) during the epidemiological surveys;
- *T. evansi* stocks which were isolated from a group of buffaloes transported from Central Java to North Sumatra (14 stocks); from local buffaloes in North Sumatra (17 stocks). These stocks were collected from Central Java buffaloes before and 13 months after transportation to North Sumatra. Samples were also taken from local (North Sumatra) buffaloes at 13, 24 and 32 months intervals after the Central Java buffaloes were transported;
- Stocks which were collected from a group of Bali cattle on a feedlot in Lampung (17 stocks) on three occasions. The second visit was carried out 1 month after the first visit and the third visit 3 months after the second visit.

3.1.1. Blood Sample Collections

Blood samples were taken by jugular vein puncture for parasitological examinations and collected into 5-ml evacuated glass tubes (Vacutainers with EDTA, Becton Dickinson, Oxford, Great Britain) containing ethylenediaminetetraacetic acid as anticoagulant. The tubes containing blood samples were kept on ice until parasitological examination on the day of collection.

3.1.2. Parasitological Examination

Parasitological examinations to detect the presence of trypanosomes were carried out by microhaematocrit centrifugation technique (MHCT) described by Woo (1970). The presence of trypanosomes was carried out by examining the buffy coat (plasma/leukocyte interphase) in the glass capillary tubes at 100 magnification.

3.1.3. Mouse Inoculation

The *T. evansi* infected blood samples by MHCT examination (100-200 μl) were inoculated into mice intraperitoneally and examinations of parasitaemia in the infected mice were carried out daily. The infected mice were then transported to the laboratory at BALITVET, Bogor. Three to five days after the inoculation, the mice usually had high parasitemia. The trypanosome stabilates were prepared from high parasitaemic mouse blood.

3.1.4. Cryopreservation of Trypanosome Stocks

Stabilates were prepared from high parasitaemic mouse blood when the number of trypanosomes was >50 trypanosomes per microscope field (400x magnification). The blood was collected by heart puncture, after exsanguination of the infected mouse, into a heparinised tube (50 Unit per ml blood) and kept on ice until ready for stabilate preparation. The collected blood was mixed with PSG in a 1:1 ratio, then glycerol was added to the mixture to give the final concentration of 7.5% (v/v). The blood mixture was drawn into capillary tubes (Hawksley, London) until the capillaries were 1/3 full. The capillaries were sealed by heating both ends with gas bunsen and placed in a cryopreservation tube (NUNC) with holes made at the bottom of the tube to let the liquid nitrogen immerse into the tube. The trypanosome stock identification was recorded in the stabilate book: host details (species, breed, sex and identification number, when applicable); isolation date, place of isolation and other information on subinoculation details before storage as stabilates, methods for cryopreservation and parasite motility and infectivity after cryopreservation. Stabilates identification, designated as BAKIT number, in the liquid nitrogen was recorded by

inserting a piece of paper with the stabilate number according to the record book. The cryopreservation tube (NUNC) containing stabilate capillaries was then placed in a self made polystyrene box and kept in a deep freezer (-70^0 C) overnight before final transfer into the liquid nitrogen tank for storage.

3.2. PREPARATION OF *T. EVANSI* DNA EMBEDDED IN AGAROSE

The preparation of *T. evansi* DNA embedded in agarose involves several steps, including subinoculations of a cryopreserved stabilate to develop parasitaemia, separation of trypanosomes from the blood cells and liberation of DNA molecules from the cell intact.

3.2.1. Trypanosome Populations Expansion

A cryopreserved population of *Trypanosoma evansi* was expanded by intraperitoneal inoculation into two TO male mice (weigh 20-25 grams). Three days later, when the parasitaemia reached more than 100 trypanosomes per microscope field (x 400 magnification) the mice were exsanguinated. The infected mouse blood (0.5 ml) was then subinoculated intraperitoneally into each of two Wistar male rats (weigh 150-200 grams). Three or four days later, when a high parasitaemia was achieved (>100 trypanosomes/microscope field, at x400 magnification), the rats were exsanguinated under halothane anaesthesia and the infected rat blood was collected by heart puncture. The infected blood was transferred into a universal container and kept on ice until required.

3.2.2. Separation of Trypanosomes from the Blood Cells

Trypanosomes were separated from the blood cells according to the method described by Lanham and Godfrey (1970) by passing through a Diethylaminoethyl cellulose (DEAE 52, Whatman Biochemicals Kent, England) column which had been equilibrated with PSG and adjusted to the pH 8.0 with 5% orthophosphoric acid. The trypanosomes were collected into a 150 ml tube and kept on ice; then transferred into some universal containers. Collected parasites in PSG were centrifuged at 3,000 rpm for 15 minutes at 4^0 C and the supernatant was discarded. Trypanosomes were washed three times with PSG by centrifugation at 3,000 rpm for 15 minutes at 4^0 C.

3.2.3. Counting the Number of Trypanosomes in PSG

The trypanosome pellet obtained after washing was diluted to 1 ml with PSG. Serial dilutions of trypanosomes in PSG (from 1:10 to 1:10,000) were prepared. The number of trypanosomes was counted starting from the lowest concentration of the dilution using the

Neubauer counting chambers (Hawksley, London). The number of parasites was calculated as described by Dacie and Lewis (1980) for white blood cells. On occasions further dilutions were prepared when the high density of trypanosomes is observed in the counting chamber.

3.2.4. Preparation of Agarose-Embedded Trypanosome Blocks

The agarose embedded DNA of *T. evansi* were prepared according to the method described by Van der Ploeg, Gottesdiener, Korman *et al.* (1992). Latex gloves were used throughout all stages of preparing agarose embedded DNA from the trypanosome pellets. All buffers used were sterilised by either autoclaving or filter sterilising using 0.2 μm pore size filters (Sartorius, UK). All equipment used was either autoclaved or swabbed with 70% alcohol (such as spatulas and cover slips to cut the gel blocks).

The tube containing the washed trypanosome pellet was transferred to a waterbath held at 50^0 C. In a typical experiment, 0.5×10^9 trypanosomes/ml was mixed gently with 5 ml of 1.3% of molten low melting point (LMP) agarose (Beckman, GeneLine) in sterile NET buffer (100 mM EDTA, 20 mM NaCl and 10 mM Tris, pH 8.0) to give a final concentration of 10^8 trypanosomes/ml. All manipulations were carried out at 50^0 C and used sterile wide bore pipette tips. The mixing of trypanosomes with the LMP agarose was carried out gently using large-bore pipette tips to prevent shearing of the DNA.

The Beckman GeneLine TAFE system comes complete with sample moulds designed to produce agarose blocks; two GeneLine® II sample moulds, made of perspex glass, comprising of 5 wells per mould (2 x 1 x 0.1 cm per well). The trypanosomes-LMP agarose mixture was then carefully pipetted into the gel moulds at a volume of 200 μl per well. The agarose filled GeneLine® II moulds were then allowed to solidify at 4^0 C for 1 hour. When set, the agarose blocks were removed from the moulds and transferred into sterile petri dishes containing 10 ml of ET buffer pH 8.0 (50 mM EDTA disodium dihydrate, 1 mM Tris). Each agarose block was cut into 10 pieces of approximately 4x5x1 mm, using sterile cover slips. Agarose blocks containing trypanosomes were digested with digestion buffer consisting of 0.5 M EDTA disodium dihydrate, 1% sodium dodecyl sulfate and either 1 mg/ml Pronase E (Sigma Chemical, St. Louis, USA) or 0.5 mg/ml Proteinase K (Sigma Chemical, St. Louis, USA). The petri dishes containing the agarose blocks were floated in a 50^0 C waterbath and digested for a period of three days; digestion buffers were replaced daily with fresh buffer. After the digestion was completed, the agarose blocks were washed three times (30 minutes for each wash), by gentle shaking at room temperature, with 10 ml of ET buffer pH 8.0 (50 mM EDTA and 1 mM Tris). The blocks were then washed a further three times (30 minutes for each wash) using 10 ml of TE buffer pH 7.4 (10 mM Tris and 1 mM EDTA), by gentle shaking at room temperature, and the buffer was replaced with the fresh

buffer each wash. The agarose blocks were stored at 4^0 C in sterile 5 ml bijous containing 3 ml TE buffer (pH 7.4) until required for electrophoresis.

3.3. BIOIMAGE® ANALYSIS

BioImage ® Whole Band Analyser computer software package (Millipore, USA) was used throughout the study for determination of the band sizes and comparison of the banding patterns. The band sizes were determined by comparison with the components of an appropriate standard marker preparation (lambda DNA or *Saccharomyces cerevisiae* for karyotype analysis and 1 kb DNA ladder for RAPD analysis, Riboprinting and SSR-PCR). Using BioImage® Whole Band Analyser, the particular banding pattern of each stock was compared to that of each other stock and their degree of similarity calculated according to the number of common bands between any 2 stocks divided by the total number of uncommon bands present in both stocks (BioImage®, Millipore, USA). Stocks with (>10% similarity) in their banding patterns were then grouped together and the final result of the analysis was presented as a dendrogram. Numerically analysis can be represented as: Percentage of similarity = $[a \div \{(x-y)-a\}]$ 100% where a is the number of bands shared by both stocks compared; x is the number of bands separated in the first stock; y is the number of bands in the second stock (Gibson *et al.*, 1980). All calculations of band sizes, pattern generation and similarity indices were carried out by the BioImage® Whole Band Analyser computer software (Millipore, USA).

CHAPTER FOUR

CHARACTERISATION OF *TRYPANOSOMA EVANSI* BY PULSED FIELD GEL ELECTROPHORESIS

4.1. INTRODUCTION

Several approaches have been used for the identification and differentiation between trypanosome populations that affect animals and humans. Criteria such as morphology, behaviour in an insect vector, and host preferences have previously been used to classify trypanosomes (Hoare, 1972). These approaches were not entirely satisfactory and subsequently immunological, biochemical and molecular approaches for trypanosome characterisation were therefore investigated.

One molecular technique that offered accurate detection of differences in the number and size of chromosomes among various protozoa was pulsed field gel electrophoresis (PFGE). The development of PFGE allowed major contributions to studies on the genomes of parasitic protozoa. Pulsed-field gel electrophoresis, which was first applied to separate yeast chromosomal DNA (Schwartz and Cantor, 1984), made it possible to establish karyotypes and the chromosomal organisation of *T. brucei* (Van der Ploeg *et al.*, 1984a). Molecular karyotype analysis showed chromosome size polymorphism among different stocks of trypanosomes (Van der Ploeg *et al.*, 1984c; Gibson and Borst, 1986; Boid *et al.*, 1992) and *Leishmania* (Tavares *et al.*, 1992; Katakura *et al.*, 1993). Chromosome rearrangements detected in small to intermediate-sized chromosomes in *T. brucei*, were related to changes in the expression of surface antigen genes (Van der Ploeg *et al.*, 1984c). There is evidence, however, that the karyotype of *T. congolense* is stable following transmission in mice, cattle, goats and tsetse fly (Masake *et al.*, 1988) and this karyotype analysis can be used for epidemiological studies.

Pulsed-field gel electrophoresis has been applied for grouping closely related species and for the identification of new field isolates in epidemiological studies with *Leishmania* (Samaras and Spithill, 1987; Saravia, Weigle, Segura, *et al.*, 1990; Katakura *et al.*, 1993). It

was shown that strains of *Leishmania* isolated from the same geographical areas exhibited minor polymorphisms in their karyotype, whereas strains from different geographical areas showed definite differences in their karyotypes (Samaras and Spithill, 1987).

Analysis of the molecular karyotypes of *T. evansi* stocks showed limited heterogeneity in the karyotype patterns in 42 stocks isolated from camels in Kenya (Waitumbi & Young, 1994) and 12 stocks isolated from buffaloes, horses, mules and camel in China (Lun *et al.*, 1992b). It was suggested that karyotype homogeneity among the *T. evansi* stocks in Kenya might be related to the rigorous application of trypanocidal drugs (Waitumbi *et al.*, 1994), since karyotype heterogeneity was observed in *T. evansi* stocks isolated from camels kept in an area where less trypanocidal drug had been used (Waitumbi and Young, 1994). The homogeneity among the field isolates from areas where the chemotherapy regimens had been applied may infer the presence of drug resistant trypanosomes (Waitumbi *et al.*, 1994). The karyotype patterns of *T. evansi* isolated in China were similar, although they were isolated from widely distributed areas in southern China, however, a stock isolated from the far northwest of China had a different karyotype pattern (Lun *et al.*, 1992b).

This chapter describes; firstly the optimisation of sample preparation for PFGE based on the method described by Van der Ploeg *et al.* (1984a; 1992). Various trypanosome concentrations and enzymes included in the agarose block preparations to establish the optimal conditions for resolution of chromosomal banding patterns of *T. evansi.*

The second part of this chapter describes the application of Transverse alternating field electrophoresis (TAFE) system (Gardiner, 1992) to analyse the karyotypes of 80 *T. evansi* stocks collected from infected cattle, buffaloes and horses in 10 geographically separated areas of Indonesia. This study was carried out to determine the distribution of karyotype polymorphism and the potential epidemiological significance of any karyotype differences. This chapter also describes the analysis of molecular karyotypes of stocks collected from transported buffaloes from Central Java to North Sumatra. This study was carried out to determine whether karyotyping could be used to determine if there was any introduction of different type of trypanosome with different pathogenicity from transported (Central Java) to local (North Sumatra) buffaloes or vise-versa. Molecular karyotype analysis was also carried out in stocks isolated from a group of Bali cattle kept together in a feedlot in Lampung experiencing an outbreak of infection. For comparison purposes, karyotype patterns of *T. brucei* and *T. congolense* were also determined alongside the *T. evansi* stocks as outliers.

The third part of this chapter describes the expanded separation of chromosomal DNA in an attempt to maximise the characterisation of the *T. evansi* genome. This work focuses on nine *T. evansi* stocks representing 9 different karyotypes were subjected to the expanded TAFE conditions to resolve chromosomal bands into different size classes: 0-100 kb., 50-300 kb, 50-500 kb, 50-900 kb and 3.5-5.7 Mb.

The fourth part of this chapter describes the comparison of the karyotype patterns between *T. evansi* stocks that are resistant and sensitive to trypanocidal drugs. The aim of this study was to investigate whether karyotyping could be used to distinguish between drug sensitive and drug resistant stocks of *T. evansi*, specifically for suramin and cymelarsan.

The fifth part of this chapter describes the molecular karyotyping among relapse populations in an attempt to establish a possible cause of the karyotype polymorphisms seen between *T. evansi* stocks in Indonesia. Two separate studies were carried out to determine the relationships between antigenic variations and karyotype polymorphism in relapse populations derived from infections with a field stock in one experiment and with a clone of the same stock in the second experiment.

The sixth part of this chapter describes methods used to try to extract DNA from a 200 kb fragment from a 1% agarose TAFE gel. This study was carried out in an attempt to develop a method to produce a chromosome probe that could be used to identify related *T. evansi* stocks from different areas of Indonesia.

The seventh part of this chapter present results on the characterisation of stocks on the basis of the chromosomal location of six gene probes: phospholipase C, Cysteine Proteinase, *T. brucei* rRNA coding region, *T. brucei* tubulin, Aldolase and *T. brucei* glucose-6-phosphate isomerase. This study was carried out to provide further information on the karyotype variability of *T. evansi* stocks with reference to established functional gene markers.

4.2. OPTIMISATION OF SAMPLE PREPARATION FOR PULSED-FIELD GEL ELECTROPHORESIS

4.2.1. INTRODUCTION

Before PFGE the chromosome-sized DNA molecules have to be liberated intact from the cell and disentangled before separation by the PFGE (Van der Ploeg *et al.*, 1984a). In this experiment, agarose block preparation was based on the method described by Van der Ploeg *et al.* (1984a; 1992). The lysis and deproteinisation were carried out on trypanosomes embedded in agarose as had been suggested by Schwartz and Cantor (1984) because of the large size of DNA involved, the agarose protects the DNA against shearing.

Combinations of different enzymes and different trypanosome concentrations were investigated for the best resolution of DNA fragments. It has been suggested that the optimal concentration of trypanosomes contained in the agarose blocks might differ between

trypanosome species because of differences in the DNA contents (Gibson and Miles, 1986). In order to establish the optimal conditions for resolution of chromosomal bands, various trypanosome concentrations and the composition of enzymes added in the preparation of the agarose blocks were tested.

4.2.2. MATERIALS AND METHODS

4.2.2.1. Trypanosoma

A cryopreserved population of *Trypanosoma evansi* (TREU 2265), originally isolated from a buffalo in Tuban, East Java, was used in the present study.

4.2.2.2. Preparation of Agarose-Embedded Trypanosome Blocks

After the separation of trypanosomes from blood cells, the tube containing the washed trypanosome pellet was transferred to a waterbath held at 50^0 C, and the parasite concentration adjusted to 10^{10} trypanosomes/ml by the addition of an appropriate volume of 1.3 % of molten low melting point (LMP) agarose (Beckman, GeneLine) in sterile NET buffer (100 mM EDTA, 20 mM NaCl and 10 mM Tris, pH 8.0). In a typical experiment, 5 x 10^9 trypanosomes/ml was mixed gently with 0.5 ml of 1.3 % of molten LMP agarose to give a final concentration of 10^{10} trypanosomes/ml. All manipulations were carried out at 50^0 C and used sterile wide bore pipette tips. A set of 5-10 fold dilutions was prepared from the 10^{10} trypanosomes/ml material by adding appropriate volumes of 1.3% of molten LMP agarose. All the dilution procedures were carried out in a waterbath held at 50^0 C to keep the LMP agarose liquid. The mixing of trypanosomes with the LMP agarose was carried out gently using large-bore pipette tips to prevent shearing of the DNA.

Two GeneLine® II sample moulds, made of Perspex, comprising of 5 wells per mould (2 x 1 x 0.1 cm per well) were used. The experimental strategy for optimising block preparation is presented in **Figure 4.1.** The trypanosomes at concentrations of 10^{10}, 10^9, 10^8, 10^7 and 10^6 trypanosomes/ml were then carefully pipetted into the gel moulds at a volume of 200 μl per well. Two agarose- embedded sample blocks were prepared from each trypanosome dilution. The agarose filled GeneLine® II moulds were then allowed to solidify at 4^0 C for 1 hour. When set, the agarose blocks were removed from the moulds and transferred into sterile petri dishes (one for each different trypanosome concentration) containing 10 ml of ET buffer pH 8.0 (50 mM EDTA disodium dihydrate, 1 mM Tris).

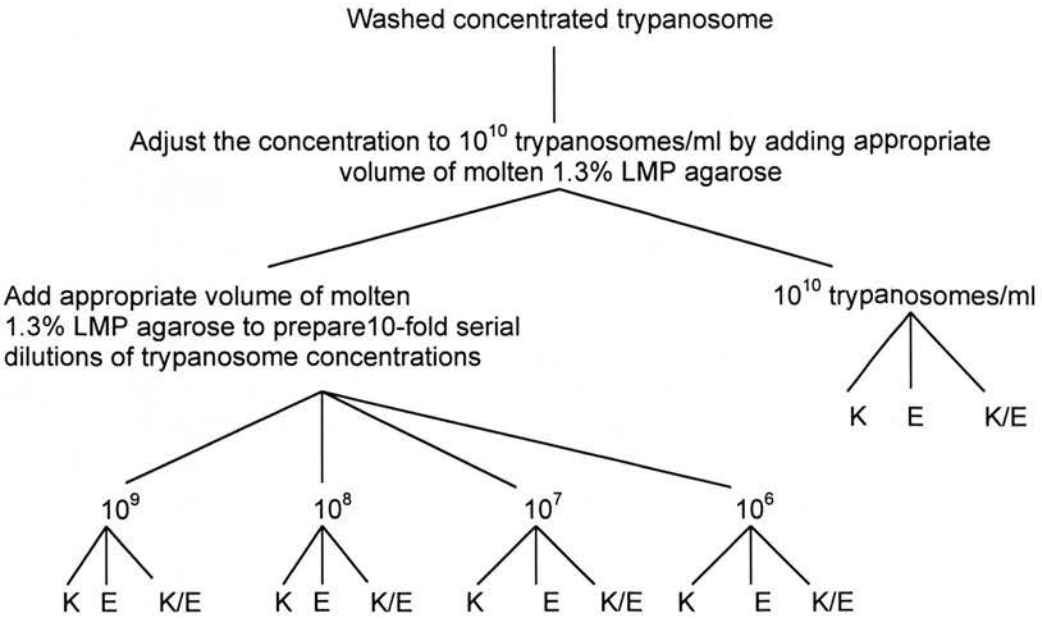


Figure 4.1. Experimental strategy for optimising agarose block preparation. K = Protease K; E = Pronase E; K/E = Protease K and Pronase E.

Each agarose block was cut into 10 pieces of approximately 4x5x1 mm, using sterile cover slips. Each trypanosome dilution yielding 20 agarose sample blocks was transferred into 3 further petri dishes, each containing 6 or 7 blocks. Each petri dish contained 10 ml of one of three digestion buffers.

Agarose blocks containing trypanosomes at each concentration were digested separately with each of the 3 different digestion buffers. Trypanosomes were digested at 50^0 C over a period of 3 days. Three digestion buffers were used all consisting of 0.5 M EDTA disodium dihydrate, 1% sodium dodecyl sulfate and either 1 mg/ml Pronase E (Sigma Chemical, St. Louis, USA), 0.5 mg/ml Proteinase K (Sigma Chemical, St. Louis, USA) or both 1 mg/ml Pronase E and 0.5 mg/ml Proteinase K. The agarose blocks were digested for three days as described in Chapter 3, Section 3.2.4.

Figure 4.2. GeneLine™ II DNA Mapper. Source: The GeneLine™ II DNA Mapper (Beckman Bulletin No. 7863).

4.2.2.3. Transverse Alternating Field Electrophoresis

A commercially produced TAFE pulsed field gel electrophoresis system, GeneLine® II, DNA Mapper (Beckman Instruments) (**Figure 4.2**) was used throughout this work for the separation of *T. evansi* chromosomal DNA. The equipment consists of: a) Electrophoresis tank with two pairs of electrodes; b) Buffer cooling system; c) Programme controller; d) Electrophoresis power supply.

The electrophoresis tank is connected with the buffer cooling system that circulates the electrophoresis buffer from the tank into the heat exchanger, where it is cooled as it passes through the coiled plastic tubing that is immersed in the refrigerated waterbath, then returns to the tank. The buffer flow maintains appropriate temperature (14 ± 0.5^0 C) throughout the electrophoresis runs.

The electrophoresis chamber is also connected to a power supply via a programme controller unit that can store up to 99 programmes, with a maximum of 120 separate stages, and capable of controlling the switch intervals from 1 second to 99 hours and running duration of maximum 100 hours. The programme control unit can be set up to 500 mA, when a constant current is used or 500 Volts, when a constant voltage is used.

4.2.2.4. Gel Preparation

The electrophoresis buffer (TAFE buffer) was prepared as a 20x stock solution consisting of 0.01 M EDTA free acid, 0.45 M Tris, 0.45 M Boric acid which was sterilised by autoclaving at 15 lb square inch for 15 minutes before use. The electrophoresis buffer was prepared immediately prior to use by diluting 1 part of the 20x TAFE buffer with 19 parts of 18.2 MΩ water.

Latex gloves were worn at all times during the preparation and loading of the gel. To prepare a standard 15x15x0.75 cm agarose gel requires the gel frame, gel mould plate and gel mould sheet. All components were cleaned with 18.2 MΩ water before assembling them. The sample comb was sterilised by rinsing with 70 % ethanol before use. The gel moulding equipment was assembled according to the manufacturer's recommendation as shown in **Figure 4.3 (step 1 and 2)**. After assembly, the gel mould was placed on a level surface.

For most electrophoretic runs gels were prepared using 1% (w/v) low electroendoosmosis (LE) agarose (Beckman). Each gel required a total volume of 180 ml of the 1% (w/v) agarose. The required quantity of agarose (1.8 g) was added to 180 ml of 1x TAFE buffer previously prepared from the 20x TAFE stock by the addition of an appropriate quantity of 18.2 MΩ water. The agarose suspension was then melted in a microwave for 3½ minutes at

maximum 500W setting with an occasional gentle swirling of the bottle. After the agarose was completely dissolved, the solution was cooled to approximately 50^0 C before pouring into the prepared gel mould. The gel was poured carefully, avoiding any air bubbles **(Figure 4.3 step 3)** and then the sample comb was placed in the gel frame slot. After pouring, the gel was left to cool at room temperature for approximately 30 minutes then transferred to a refrigerator and allowed to set for 1 hour at 4^0 C.

4.2.2.4.1. Sample loading

Approximately 4 litres of remaining electrophoresis buffer was poured into the electrophoresis tank. The system was assembled and the buffer cooler turned on to chill the buffer to 14^0 C before the electrophoretic run was commenced.

The gel slab was removed from the refrigerator and the sample comb was carefully removed **(Figure 4.3 step 4)**. The samples were inserted into the front part of sample wells using a sterile small spatula **(Figure 4.3 step 5)**. Care was taken to avoid introducing air bubbles in between the sample and the gel.

After loading all samples into the sample wells they were sealed with 1 % LMP agarose in 1x TAFE buffer **(Figure 4.3 step 6)**. The gel was then returned to the refrigerator (4^0 C) for a further 30 minutes.

The masking tape was carefully removed from the gel mould **(Figure 4.3 step 7)** and then the gel frame containing the gel and samples was removed from the mould by separating the gel frame from the gel mould frame and the gel mould sheet **(Figure 4.3 step 8 and 9)**. The gel frame containing the agarose gel was then placed vertically into the centre slot of the electrophoresis tank and the lid placed in position after connecting the power supply to the electrophoresis tank via the programme controller.

4.2.2.4.2. Running the gel

The connecting block from the controller was attached to the lid. The electrophoresis consisted of three stages, all run at a constant current at 275 mA, with a total electrophoresis time of 24 hours. The first stage was run at 15 sec pulse time with electrophoresis duration of 8 hours, the second stage used 30 sec pulse time and run for 12 hours and the third stage was run at 15 sec pulse time for 4 hours. According to published work this strategy should resolve bands in the 50-500 kb size range. The temperature was maintained at a constant 14^0 C during the electrophoretic run.

At the end of the programme the system was switched off, the lid removed and the gel frame removed from the gel tank.

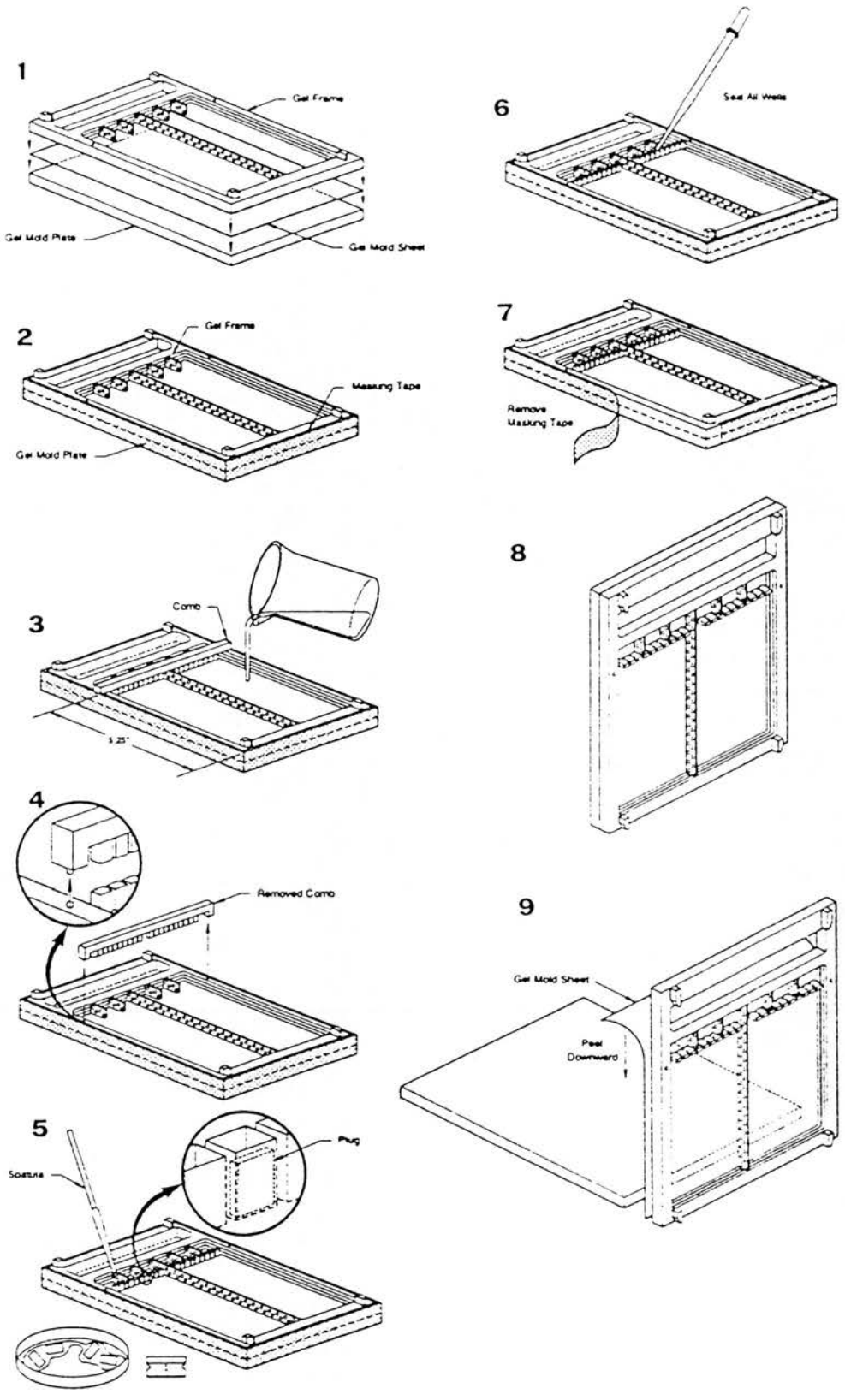


Figure 4.3. Gel preparation for TAFE. Source: The GeneLine™ II System. Operating Instructions (Beckman Instructions 015-248496, 1991).

4.2.2.4.3. Gel staining and destaining

The gel in the frame was removed from the chamber and transferred to the staining tank containing 300 ml distilled water containing 0.5 μg/ml ethidium bromide. The gel was stained at room temperature for 1 hour and then destained in approximately 500 ml of distilled water over a period of 3 hours. The water used to destain the gel was replaced with fresh distilled water after the first 1 hour.

4.2.2.4.4. Visualisation of the TAFE separated Chromosomal DNA

The destained gel was visualised by placing on a 254 nm UV transilluminator. The DNA appeared as pinkish fluorescence against a dark background. Each gel was photographed using an MP4 Polaroid land camera and Type 55 positive/negative Polaroid film. The film was exposed at f 4.5 through a red filter (Wratten 23A) for 45 seconds. Both a positive print and a negative were obtained. The negative of the gel photograph was scanned over a white light via Kodak Megaplus Model 1.4 camera interfaced with the BioImage® Whole Band Analyser (Millipore, USA). The DNA band sizes in kilobasepairs (kb) were calculated by comparing the band sizes in the lambda DNA standard marker included in every gel using the BioImage ® Whole Band software linear regression method.

4.2.3. RESULTS

4.2.3.1. Separation of Standards

The PFGE using a 3 stage run over a 24 hours period clearly separated 12 bands in the lambda ladder-DNA size standard. The 12 bands were resolved linearly between the size range of 48.5 to 582.0 kb (r^2 = 0.97). The bands over 600 kb in the size standard were not as clearly resolved by the running conditions (**Figure 4.4**). The reciprocal of distance of the band mobility of up to 600 kb is linear with the band size (r^2 = 0.97).

4.2.3.2. Separation of Trypanosoma evansi

4.2.3.2.1. Effect of different *T. evansi* concentration in agarose blocks

The banding patterns of *T. evansi* in agarose blocks containing 5 different trypanosome concentrations separated by PFGE were shown in **Figure 4.4**. Tracks 3, 4 and 5 in **Figure 4.4** were each loaded with an agarose block containing 10^{10} trypanosomes per ml (equivalent to $2x10^8$ trypanosomes per sample well). Tracks 6, 7 and 8 in **Figure 4.4** were

each loaded with an agarose block containing 10^9 trypanosome per ml, which equivalent to $2x10^7$ trypanosomes per sample well. Each sample well in tracks 10, 11 and 12 in **Figure 4.4** were loaded with an agarose block containing 10^8 trypanosomes/ml (or equivalent to $2x10^6$ trypanosomes/ sample well); tracks 14, 15 and 16 with 10^7 trypanosomes/ml (equivalent to $2x10^5$ trypanosomes/sample well) and tracks 16, 17 and 18 with 10^6 trypanosomes/ml, which equivalent to 10^4 trypanosomes per sample well. In general it was observed that the fluorescent background reduced significantly with decreasing concentrations of trypanosome in agarose blocks. The banding patterns were mostly clear in the blocks prepared from 10^8 trypanosome/ml or equivalent to $2x10^6$ trypanosomes per sample well (**Figure 4.4 lane 10-12**) with clear separation and low background of fluorescence. Ten DNA bands with different intensities were observed in the size range of 86 kb to 1.3 Mb.

A high background fluorescence was detected in the banding patterns of the agarose blocks containing 10^9 and 10^{10} trypanosome/ml or, equivalent to $2x10^7$ and $2x10^8$ trypanosomes per sample well respectively (**Figure 4.4 lane 6-8 and lane 3-5 respectively**). Ten DNA bands were observed in the size range of 86 kb to 1.3 Mb in the agarose blocks containing 10^9 trypanosome/ml. The agarose blocks prepared from 10^{10} trypanosome/ml, however, did not show any clear banding patterns and the number of bands was difficult to determine because of the background staining was too high (**Figure 4.4 lane 3-5**).

Lower trypanosome concentration than 10^8 trypanosome/ml (10^7 and 10^6 trypanosome/ml), however, did not show any clear banding patterns (**Figure 4.4 lane 14-18**). Four faint bands were observed between 86 kb to 1.3 Mb in the blocks containing 10^7 trypanosomes/ml than those observed in the blocks containing 10^8 trypanosome/ml and the presence of bands were not observed in the blocks containing 10^6 trypanosomes/ml. The presence of DNA was detected in the gel slot containing the agarose blocks prepared from 10^7 trypanosome/ml and fluorescence was not observed in the gel slot containing 10^6 trypanosome/ml.

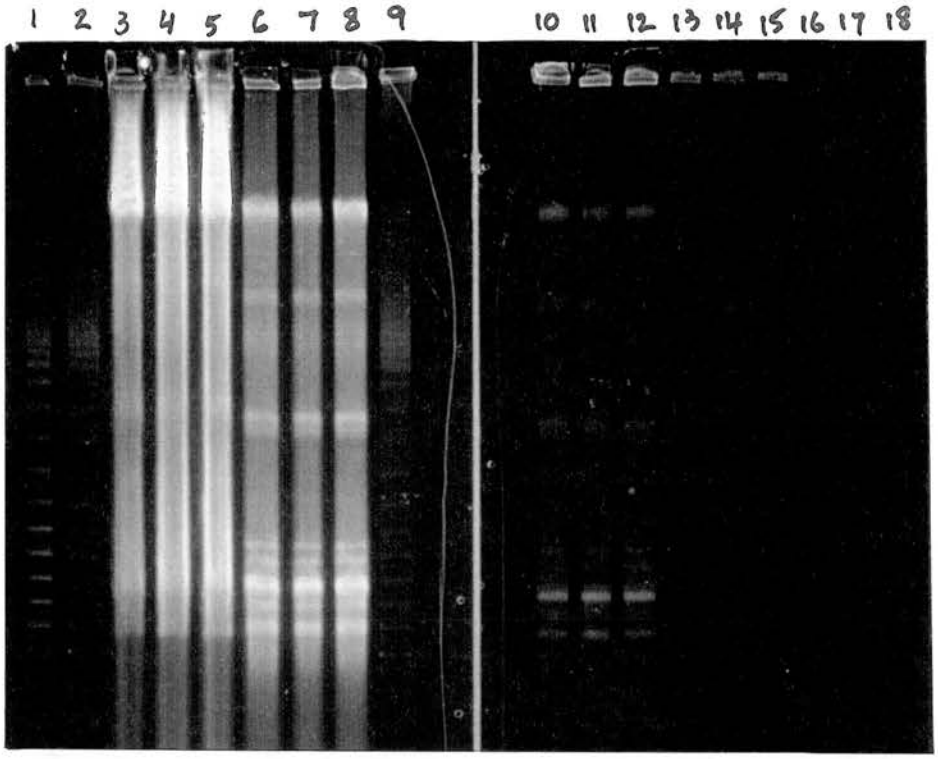
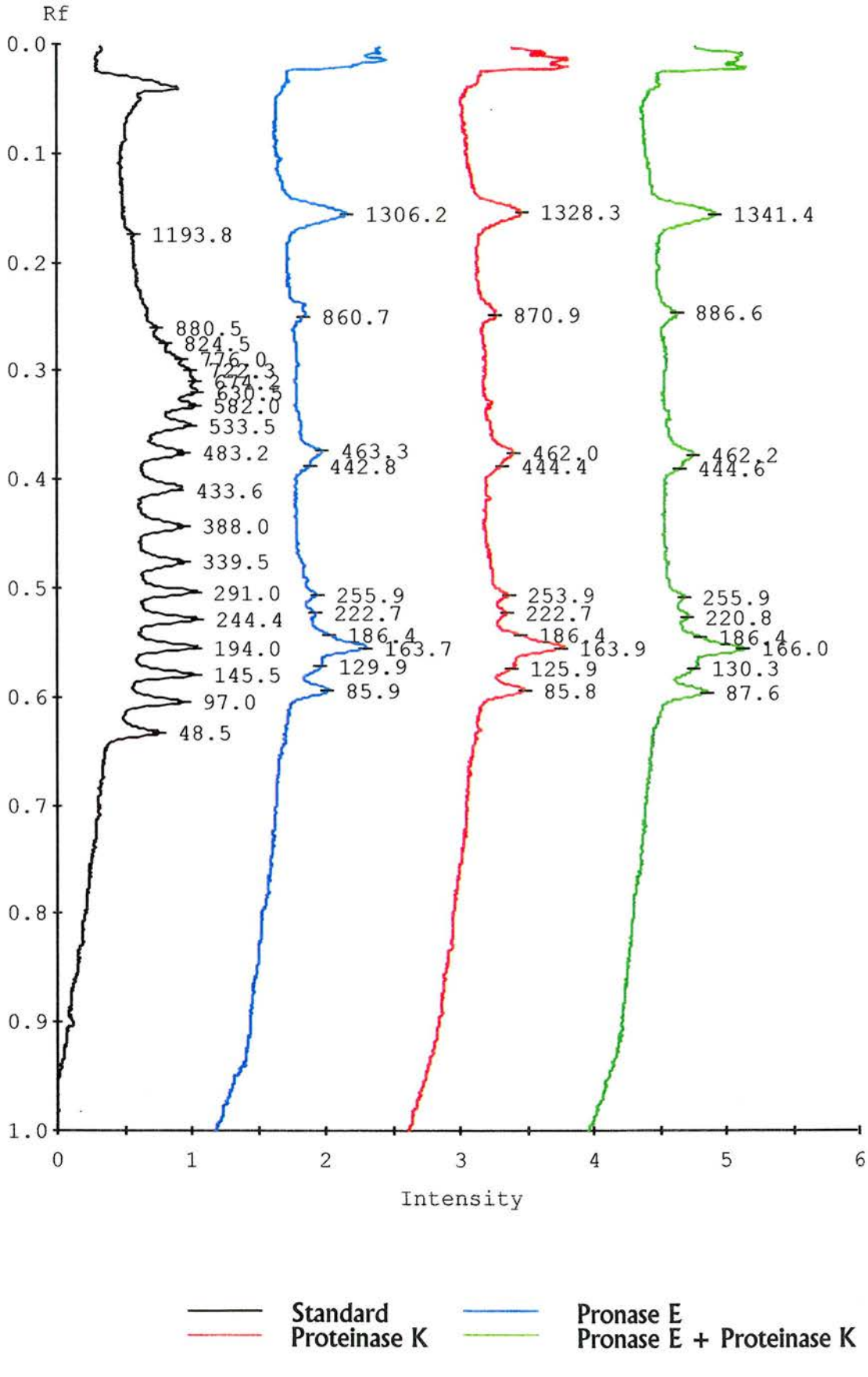


Figure 4.4. The effect of changes in trypanosome concentration and digestion buffers used in the preparation of agarose embedded DNA.

Tracks 1, 2, 9: Lambda DNA concatemer standard marker
Track 3: DNA from $2x10^8$ trypanosomes digested with Protease K and Pronase E.
Track 4: DNA from $2x10^8$ trypanosomes digested with Protease K.
Track 5: DNA from $2x10^8$ trypanosomes digested with Pronase E.
Track 6: DNA from $2x10^7$ trypanosomes digested with Protease K and Pronase E.
Track 7: DNA from $2x10^7$ trypanosomes digested with Protease K.
Track 8: DNA from $2x10^7$ trypanosomes digested with Pronase E.
Track 10: DNA from $2x10^6$ trypanosomes digested with Protease K and Pronase E.
Track 11: DNA from $2x10^6$ trypanosomes digested with Protease K.
Track 12: DNA from $2x10^6$ trypanosomes digested with Pronase E.
Track 13: DNA from $2x10^5$ trypanosomes digested with Protease K and Pronase E.
Track 14: DNA from $2x10^5$ trypanosomes digested with Protease K.
Track 15: DNA from $2x10^5$ trypanosomes digested with Pronase E.
Track 16: DNA from $2x10^4$ trypanosomes digested with Protease K and Pronase E.
Track 17: DNA from $2x10^4$ trypanosomes digested with Protease K.
Track 18: DNA from $2x10^4$ trypanosomes digested with Pronase E.

Figure 4.5. Lane profiles of the *T. evansi* DNA embedded in agarose blocks prepared from $2x10^6$ trypanosomes and digested with three digestion buffers, each containing a) Pronase E; b) Protease K; c) Protease K and Pronase E.


Rf
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1193.8
880.5
824.5
776.0
722.3
674.2
630.5
582.0
533.5
483.2
433.6
388.0
339.5
291.0
244.4
194.0
145.5
97.0
48.5
1306.2
860.7
463.3
442.8
255.9
222.7
186.4
163.7
129.9
85.9
1328.3
870.9
462.0
444.4
253.9
222.7
186.4
163.9
125.9
85.8
1341.4
886.6
462.2
444.6
255.9
220.8
186.4
166.0
130.3
87.6
0
1
2
3
4
5
6
Intensity
Standard
Proteinase K
Pronase E
Pronase E + Proteinase K

4.2.3.2.2. Effect of different proteases

The banding pattern was identical in numbers and band intensities in all blocks prepared from equal concentration of trypanosomes and digested with three different digestion buffers. This study has shown that the type of proteases used for digesting the *T. evansi* embedded in agarose did not affect the banding pattern quality.

Three agarose blocks prepared from $2x10^6$ trypanosomes per sample well and digested each with Pronase E (lane 10, **Figure 4.4**), Proteinase K (lane 11, **Figure 4.4**) or both Pronase E and Proteinase K (lane 12, **Figure 4.4**) showed the clearest banding patterns compared to the other agarose block preparations (lane 3-8 and lane 14-18 in **Figure 4.4**). The banding patterns of these three agarose block preparations ($2x10^6$ trypanosomes per sample well) were identical in their numbers and intensities of their bands as shown in lane profiles in **Figure 4.5** produced by the BioImage® Whole Band analyser software package.

4.2.4. DISCUSSION

This study has shown that the trypanosome concentration in agarose blocks is an important factor in the final banding pattern resolution by PFGE. The study had shown that too high trypanosome concentration, which yielded too much DNA in agarose blocks, could not resolve the banding pattern and too little DNA only resulted in the disappearance of a number of bands.

It was concluded that agarose blocks containing 10^8 trypanosome/ml, which is the equivalent to 2 millions of *T. evansi* per gel sample, which is itself equal to 80 ng of *T. evansi* DNA is sufficient to yield clear banding pattern resolution by the TAFE system conditions used in this experiment. The estimation of the DNA content in agarose blocks was based on the assumption that the genome size of *T. evansi* was approximately 40 Mb containing approximately 0.04 pg per trypanosome. The blocks made from 10^{10} trypanosome/ml trypanosome which is the equivalent of 200 millions of *T. evansi* in each sample block showed high fluorescence background and the banding patterns could not be determined. The high fluorescence background is caused by too high DNA concentration present in the blocks and the poor resolution of the banding pattern separated by TAFE might be due to the presence of undigested nucleoproteins. Gardiner (1992) suggested that the high fluorescence background could be caused by the shearing of DNA due to the loading of damaged agarose blocks in the electrophoresis (Gardiner, 1992). This study, however, only used intact agarose blocks which were loaded into the gel.

The importance of optimising the trypanosome concentration in the agarose blocks for PFGE was suggested by Gibson and Miles (1986) because of the differences in the DNA contents

among different trypanosome species. Gibson and Miles (1986) prepared agarose blocks of *T. cruzi* at the concentration of 4 x 10^8 per ml, instead of 2 x 10^9 per ml for *T. brucei* (Van der Ploeg *et al.*, 1984a), because *T. cruzi* has approximately three times (0.28 pg, Lanar, Levy and Manning, 1981) the DNA content of *T. brucei* (0.09 pg, Borst *et al.*, 1982).

The differences in the DNA content may not be the only reason for optimising the trypanosome concentration in the agarose for PFGE. For example, *T. evansi* has approximately one half (0.04 pg, according to our calculation), or one quarter (0.02 pg, measured by Baker, 1961) of the DNA content of *T. brucei* (Borst *et al.*, 1982). According to our calculation, the optimal *T. evansi* concentration in agarose block for PFGE in this study would have been at least twice as high as the concentration described for *T. brucei* (2 x 10^9 trypanosomes/ml, Van der Ploeg *et al.*, 1984a). In general, Birren and Lai (1993) suggested that the optimal concentration of trypanosomes in the agarose for PFGE is ~ 1 x 10^9 cells/ml to be able to load 2-5 x 10^7 trypanosomes per lane. This trypanosome concentration is 10 to 25 times higher than the standardised agarose block preparation carried out in this study. Gibson and Borst (1986) used 2 x 10^9 trypanosome/ml in their agarose blocks for *T. brucei, T. evansi* and *T. congolense* subjected to PFGE system described by Van der Ploeg *et al.* (1984a). Our result suggests that the TAFE system used in this study for karyotyping the *T. evansi* stocks needs a lower trypanosome concentration than other PFGE systems (10^8 trypanosomes/ml or 2 x 10^6 trypanosomes per sample block), which resulted into a clear banding pattern with good resolution within straight lanes. The clear resolution and the sharpness of the bands is due to the 115^0 reorientation angle in the TAFE system. Schwartz and Cantor (1984) suggested that reorientation angles of greater than 90^0 yielded good resolution in the banding patterns and band sharpness in the PFGE. This study has shown the importance of optimising the trypanosome concentration in the agarose block according to the PFGE system employed.

Because of the large size of the DNA involved in the PFGE separation, the trypanosome protein digestion was carried out after the trypanosomes were embedded in agarose as suggested by Schwartz and Cantor (1984) for *S. cerevisiae* to protect the DNA against shearing. The nuclease activity which would degrade the intact DNA, is inhibited by maintaining the agarose blocks in a buffer containing detergent and a high concentration of EDTA. This buffer also activates the proteases reaction to digest cellular protein during inactivity of the endogenous enzymes (Birren and Lai, 1993). The washes after trypanosome digestion were carried out to release materials to diffuse out of the agarose leaving the DNA trapped in the agarose. The agarose blocks remain stable for several years because the cellular enzymes were removed completely (Birren and Lai, 1993). It was also shown that the trypanosome protein digestion using Pronase E yield as good PFGE banding pattern performance as either Proteinase K or a combination of both Pronase E and Proteinase K.

4.3. KARYOTYPE ANALYSIS OF *T. EVANSI* ORIGINATING FROM DIFFERENT AREAS OF INDONESIA

4.3.1. INTRODUCTION

Karyotype analysis of *T. evansi* stocks isolated from China (Lun *et al.*, 1992b) and Kenya (Waitumbi *et al.*, 1994) indicated the stability of the molecular karyotypes over a long period of time. The advantages of using molecular karyotyping are to study the epidemiology of *T. evansi* and dynamic of different trypanosome populations in field situations. Karyotype analysis was applied to 80 *T. evansi* field stocks isolated in widely distributed areas in Indonesia using TAFE to determine the distribution of molecular karyotype polymorphism and the significance of these karyotype differences.

4.3.2. MATERIALS AND METHODS

4.3.2.1. Trypanosomes

Trypanosoma evansi stocks were isolated from cattle, buffaloes and horses exposed to natural challenge in different areas of Indonesia. The stocks originated from animals positive by microhaematocrit centrifugation technique (MHCT) as described by Woo (1970). Blood samples from infected animals were inoculated into mice, which were transported to the laboratory at BALITVET. When the parasitaemia in each mouse reached >50/ x400 microscope field, the infected mouse was exsanguinated. The blood was collected after exsanguination of the mouse and mixed with an equal volume of PSG containing 7.5% (v/v) glycerol and cryopreserved as stabilates in liquid nitrogen at -180^0 C.

a) *Trypanosoma evansi* collected from 10 regions of Indonesia

Eighty *T. evansi* stocks (**Table 4.1**) were collected from infected cattle, buffaloes and horses during single visits in different areas in Aceh, West, Central and East Java, Madura, South Kalimantan, South and North Sulawesi and multiple visits to North Sumatra and Lampung. The stock isolation locality in widely distributed areas of Indonesia is presented in a map in **Figure 4.6**. The polymorphisms in the karyotype patterns shown by the stocks collected from the transported buffaloes in North Sumatra and from feedlot cattle in Lampung were analysed separately.

Table 4.1. *Trypanosoma* stocks used for molecular characterisation.

Area	Isolation locality (Sub District, District)	Host species	Isolate No.	Stock No.
Aceh	Timu Kemala, Pidie	Cattle	78	BAKIT 312
Central Java	Semarang	Buffalo	80	BAKIT 371
Central Java	Semarang	Buffalo	81	BAKIT 372
Central Java	Semarang	Buffalo	83	BAKIT 373
Central Java	Semarang	Buffalo	82	BAKIT 374
Central Java	Semarang	Buffalo	84	BAKIT 375
Central Java	Purworejo	Buffalo	237	BAKIT 508
Central Java	Demak	Cattle	236	BAKIT 509
Central Java	Demak	Buffalo	238	BAKIT 510
Central Java	Demak	Cattle	239	BAKIT 511
Central Java	Demak	Buffalo	242	BAKIT 512
Central Java	Purworejo	Buffalo	240	BAKIT 513
Central Java	Purworejo	Buffalo	241	BAKIT 514
East Java	Bojonegoro	Cattle	017	BAKIT 126
East Java	Sumber Salak, Banyuwangi	Cattle	201	BAKIT 467
East Java	Sumber Salak, Banyuwangi	Cattle	210	BAKIT 475
East Java	Kajar, Banyuwangi	Cattle	214	BAKIT 482
East Java	Kajar, Banyuwangi	Cattle	227	BAKIT 496
Lampung	Menggala	Cattle	148	BAKIT 409
Lampung	Menggala	Cattle	149	BAKIT 410
Lampung	Menggala	Cattle	150	BAKIT 411
Lampung	Menggala	Cattle	152	BAKIT 413
Lampung	Menggala	Cattle	154	BAKIT 415
Lampung	Menggala	Cattle	155	BAKIT 416
Lampung	Menggala	Cattle	156	BAKIT 417
Lampung	Menggala	Cattle	160	BAKIT 421
Lampung	Menggala	Cattle	170	BAKIT 431
Lampung	Menggala	Cattle	173	BAKIT 434
Lampung	Menggala	Cattle	175	BAKIT 435
Lampung	Menggala	Cattle	176	BAKIT 437
Lampung	Menggala	Cattle	178	BAKIT 439
Lampung	Menggala	Cattle	183	BAKIT 444
Lampung	Menggala	Cattle	184	BAKIT 445
Lampung	Menggala	Cattle	185	BAKIT 446
Lampung	Menggala	Cattle	200	BAKIT 463
Madura	Bangkalan	Buffalo	87	BAKIT 362
Madura	Bangkalan	Buffalo	85	BAKIT 517
Madura	Bangkalan	Cattle	145	BAKIT 519
North Sulawesi	Bolomongondow, Minahasa	Cattle	013	BAKIT 100
North Sulawesi	Airmadidi, Minahasa	Cattle	036	BAKIT 148
North Sulawesi	Tondano, Minahasa	Horse	056	BAKIT 254

North Sumatra	Airputih, Tebingtinggi	Buffalo	119	BAKIT 380
North Sumatra	Limapuluh, Tebingtinggi	Buffalo	120	BAKIT 381
North Sumatra	Airputih, Tebingtinggi	Buffalo	121	BAKIT 382
North Sumatra	Airputih, Tebingtinggi	Buffalo	123	BAKIT 383
North Sumatra	Limapuluh, Tebingtinggi	Buffalo	125	BAKIT 384
North Sumatra	Airputih, Tebingtinggi	Buffalo	126	BAKIT 385
North Sumatra	Airputih, Tebingtinggi	Buffalo	127	BAKIT 386
North Sumatra	Airputih, Tebingtinggi	Buffalo	128	BAKIT 387
North Sumatra	Airputih, Tebingtinggi	Buffalo	129	BAKIT 388
North Sumatra	Airputih, Tebingtinggi	Buffalo	130	BAKIT 389
North Sumatra	Airputih, Tebingtinggi	Buffalo	131	BAKIT 390
North Sumatra	Limapuluh, Tebingtinggi	Buffalo	133	BAKIT 392
North Sumatra	Airputih, Tebingtinggi	Buffalo	135	BAKIT 393
North Sumatra	Airputih, Tebingtinggi	Buffalo	134	BAKIT 394
North Sumatra	Airputih, Tebingtinggi	Buffalo	139	BAKIT 399
North Sumatra	Airputih, Tebingtinggi	Buffalo	140	BAKIT 400
North Sumatra	Airputih, Tebingtinggi	Buffalo	141	BAKIT 401
North Sumatra	Airputih, Tebingtinggi	Buffalo	142	BAKIT 402
North Sumatra	Airputih, Tebingtinggi	Buffalo	161	BAKIT 422
North Sumatra	Airputih, Tebingtinggi	Buffalo	162	BAKIT 423
North Sumatra	Durian, Tebingtinggi	Buffalo	163	BAKIT 424
North Sumatra	Durian, Tebingtinggi	Buffalo	164	BAKIT 425
North Sumatra	Durian, Tebingtinggi	Buffalo	165	BAKIT 426
North Sumatra	Durian, Tebingtinggi	Buffalo	166	BAKIT 427
North Sumatra	Durian, Tebingtinggi	Buffalo	167	BAKIT 428
North Sumatra	Durian, Tebingtinggi	Buffalo	168	BAKIT 429
South Kalimantan	Pengaron, Banjar	Cattle	146	BAKIT 403
South Kalimantan	Amuntai	Buffalo	198	BAKIT 461
South Sulawesi	Palangga, Gowa	Buffalo	055	BAKIT 251
South Sulawesi	Maiwa, Enrekang	Cattle	071	BAKIT 296
West Java	Ciawi, Bogor	Buffalo	034	BAKIT 134
West Java	Garut	Cattle	004	BAKIT 294
West Java	Bogor	Buffalo	229	BAKIT 498
West Java	Bogor	Buffalo	230	BAKIT 499
West Java	Bogor	Buffalo	231	BAKIT 500
West Java	Bogor	Buffalo	233	BAKIT 502
West Java	Bogor	Buffalo	232	BAKIT 503
West Java	Bogor	Buffalo	234	BAKIT 504
West Java	Bogor	Buffalo	235	BAKIT 505
Brazil (*T. evansi*)	?	Dog		TREU 2187
Kenya (*T. evansi*)	?	Camel		TREU 1810
Uganda (*T. brucei*)	?	Cattle		TREU 2177
Nigeria (*T. congolense*)	?	Cattle		TREU 2193

Figure 4.6. *Trypanosoma evansi* stocks isolation locality in 10 regions of Indonesia.

b) Trypanosoma evansi collected from transported buffaloes

Trypanosoma evansi were isolated from buffaloes transported from Semarang (Central Java) to Tebingtinggi in North Sumatra. Samples were collected in Semarang before transportation, and then 13, 24 and 32 months after the buffaloes were distributed in North Sumatra. Although the sample collections were carried out in the same study areas, none of the *T. evansi* stocks were isolated from the same animal. *T. evansi* stocks were also collected from local buffaloes located near to where the central Java buffaloes were distributed in Tebingtinggi district. Five *T. evansi* isolates from Semarang and 25 from North Sumatra were available for analysis by TAFE.

c) *Trypanosoma evansi* collected from a Bali cattle feedlot in Lampung

Seventeen *T. evansi* stocks were collected from Bali cattle in a feedlot in Lampung, South of Sumatra. The cattle kept in the feedlot originated from different areas in the provinces of Lampung and South Sumatra. Three visits were made to collect trypanosome stocks. Eight stocks were collected in the first visit in November 1991; eight stocks from visit 2, which was carried out one month later, and only one stock was collected on the third visit, in March 1992. After the collection of blood samples on the second visit, the animals were treated with suramin (Naganol®, Bayer) at a dose rate of 1 gram per 100 kg body weight.

Karyotype patterns of *T. brucei* (TREU 2177, isolated in Uganda) and *T. congolense* (TREU 2193, isolated in Nigeria) were determined alongside the *T. evansi* stocks for comparison purposes.

4.3.2.2. Transverse Alternating Field Electrophoresis (TAFE)

Agarose blocks containing *T. evansi* chromosomal DNA equivalent to 2 millions trypanosomes/block were prepared as described in Section 3.2.4. TAFE was carried out using a GeneLine II apparatus and a 4 stage electrophoresis protocol to separate chromosomal bands between 50-900 kb (Dih and Morgenstern, 1990). Stage 1 used a pulse time of 30 second for a period of 12 hrs; stage 2 used a pulse time of 90 second for a period of 12 hrs; stage 3 used a pulse time of 150 second for a period of 12 hrs and stage 4 used a pulse time of 30 minute for a period of 8 hrs. Stages 1 to 3 were run at a constant current of 275 mA and stage 4 was run at a constant current of 10 mA. Gels were stained and destained as described in section 4.2.2.4.3 and visualised as described in section 4.2.2.4.4. A 0.05-1 Mb lambda pulsed field size standard (λ ladder, BioRad, UK) was used as an internal size marker on all gels.

4.3.2.3. Karyotype Pattern Analysis

The bands' sizes were determined by comparison to the fragment sizes separated in the lambda DNA standard marker using the BioImage® Whole Band Analyser computer software package (Millipore, USA). Only chromosomal bands in the size range of 50 to approximately 1,000 kb were compared and then grouped as described in Chapter 3 (General Materials and Methods), Section 3.3.

4.3.3. RESULTS

Pulsed-field gel electrophoresis using the TAFE system separated the lambda ladder DNA size standard into 17 bands in the size range between 48.5 to 824.5 kb. The reciprocal of the distance travelled by the lambda DNA fragments of up to 824.5 kb is linear with the band size ($R^2 = 0.9909$).

The banding patterns shown by PFGE gels were stable as shown in at least two gel runs carried out under the same electrophoretic conditions. An example of the typical PFGE separation using the TAFE II system in *T. evansi* stocks originating from Indonesia is shown in **Figure 4.7**. The electrophoresis was optimised to separate DNA bands in the size range of 50 - 900 kb (following the information supplied by Beckman Ltd.). As an example, the band separation shown by BAKIT 251/North Sulawesi (lane 11 in **Figure 4.7**) had a clear resolution of banding pattern. Three sharp bands of the minichromosomes between 50-150 kb size range; nine intermediate-sized chromosomes between 150-900 kb size range, at least two large chromosomes in the compression zone and the DNAs that remained in the gel slot were observed. The general pattern of the karyotypic separation in *T. evansi* can be divided into 4 size classes: a) very large chromosomes that remained in the sample well; b) between 1 to 4 large chromosome bands (Lc) with sizes greater than 1.1 Mb, and the chromosomal bands present in the compression zone (CZ), which are not clearly resolved by this system; c) intermediate sized chromosomes (2 to 10 bands) in the size range of 150 to 900 kb; and d) small chromosomes in the size range of 50 to 150 kb. The *T. evansi* stocks studied generally contained of 1 to 4 minichromosomes bands in the 50-150 kb size range, however, in some stocks minichromosomes were not well resolved resulting in a smear of minichromosomes. Only chromosomal bands that were well resolved by PFGE were included in the analysis. Bands above 1 Mb were not well resolved even under the optimised running conditions. A poor staining of the lambda ladder was observed in lane 14 of **Figure 4.7**.

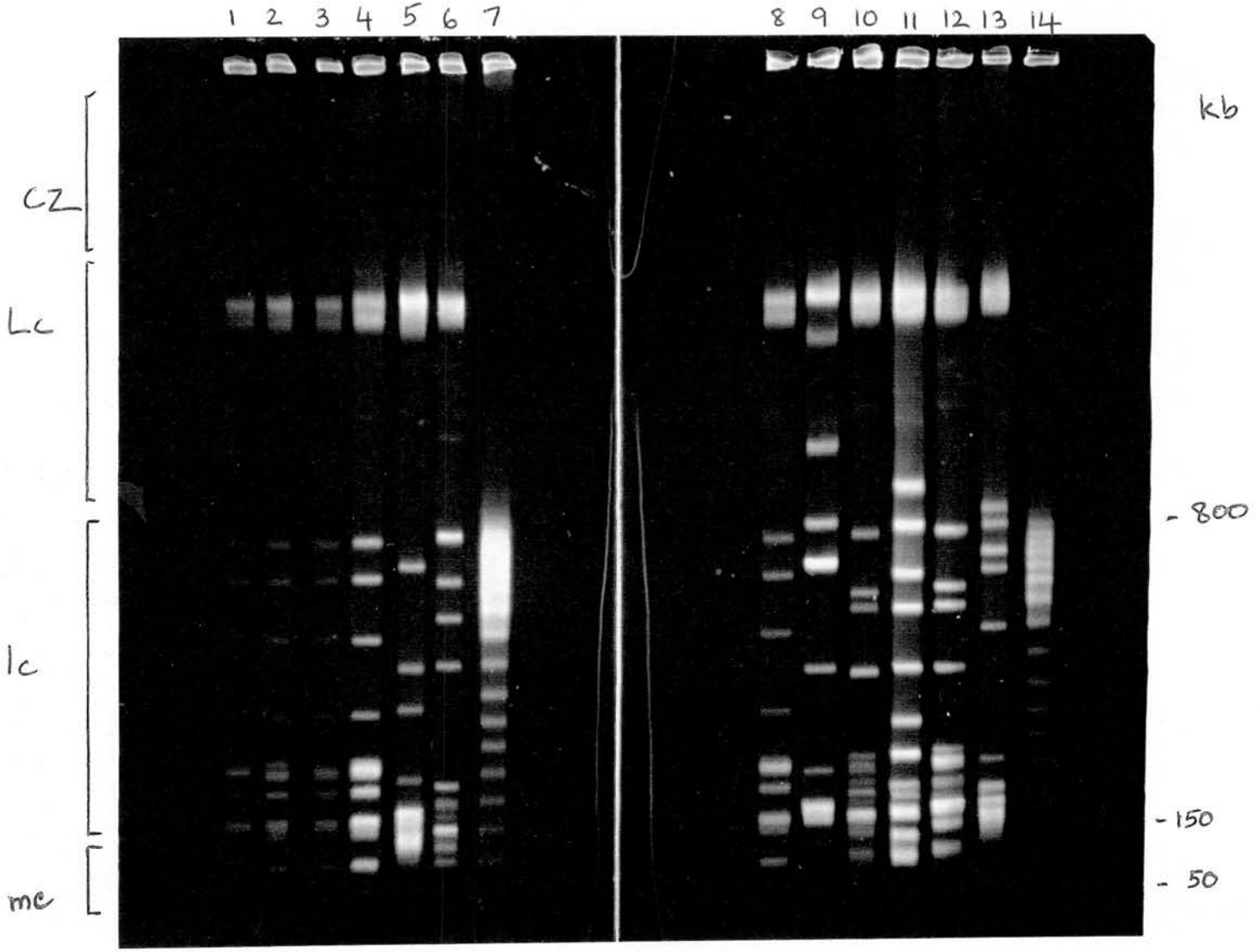

1 2 3 4 5 6 7
8 9 10 11 12 13 14
kb
CZ
Lc
lc
mc
- 800
- 150
- 50

Figure 4.7. *Trypanosoma evansi* stocks separated by Transverse Alternating Field Electrophoresis using conditions optimised to resolve DNA in the 50-900 kb size range.

Track 7, 14: Lambda DNA concatemer size standard;
Track 1: BAKIT 496/East Java;
Track 2: BAKIT 482/East Java;
Track 3: BAKIT 475/East Java;
Track 4: BAKIT 467/East Java;
Track 5: BAKIT 461/S. Kalimantan;
Track 6: BAKIT 403/S. Kalimantan;;
Track 8: BAKIT 312/Aceh;
Track 9: BAKIT 296/S. Sulawesi;
Track10: BAKIT 254/N. Sulawesi
Track11: BAKIT 251/S. Sulawesi;
Track12: BAKIT 148/N. Sulawesi;
Track13: BAKIT 100/ N. Sulawesi;.

CZ = compression zones;
Lc = Large chromosomes (>1,000 kbp);
Ic = Intermediate sized chromosomes (150-900 kbp);
mc = Minichromosomes (50-150 kbp).

4.3.3.1. Analysis of Karyotype Patterns Polymorphism in T. evansi *Stocks from Indonesia*

Among the 80 stocks tested from different areas in Indonesia, the chromosomal profiles of *T. evansi* stocks consisted of karyotype patterns 1 to 46 (**Figure 4.8**).

The karyotype group 1.1 consists of 6 stocks, which belonged to pattern 1-5, collected from Central Java (BAKIT 371, 373, 375), West Java (BAKIT 134/Suramin resistant and BAKIT 505) and a stock from North Sumatra (BAKIT 386). Two stocks from Central Java (BAKIT 373 and 375) have identical karyotypes. The 6 stocks belonged to this group have 9-12 chromosomal bands within the size range of 70 to 900 kb.

The stocks belonging to karyotype group 1.2 have 6-11 chromosomal bands between the size range of 50 to 950 kb. The karyotype group 1.2 consisting of 21 stocks with 9 stocks from Central Java (BAKIT 374, 508, 509, 512, 513, 514) and West Java (BAKIT 498, 499,502) have identical patterns, which belong to pattern 6. Karyotype pattern identity was also observed in karyotype pattern 7 consisting of 2 stocks from Lampung (BAKIT 435, 437), pattern 8 consisting of 2 stocks from North Sumatra (BAKIT 426, 428) and pattern 10 consisting of the 3 stocks isolated from an outbreak in Madura (BAKIT 362, 517, 519). Other stocks included in this group are BAKIT 511/central Java (pattern 9), 251/South Sulawesi (pattern 11), 403/South Kalimantan (pattern 12), 148/North Sulawesi (pattern 13) and 254/North Sulawesi (pattern 14).

Karyotype group 1.3 has 6-11 chromosomal bands within the size range of 80 to 850 kb and consist of 15 stocks which were assigned to pattern 15 to17. The 13 *T. evansi* stocks isolated in Lampung (BAKIT 410, 411, 413, 415, 416, 417, 421, 434, 439, 444, 445, 446, 463) have identical banding patterns (pattern 17). Other stocks included in this group are BAKIT 385/North Sumatra (pattern 15) and BAKIT 100/North Sulawesi (pattern 16).

Karyotype group 1.4, has 6-12 bands present in the size range between 45 to 990 kb and consists of 13 stocks which were all collected from North Sumatra. Two sets of identical patterns in the stocks belonging to this group were noted: BAKIT 380 and 382 have identical patterns (pattern 18) and the karyotypes of BAKIT 383, 401, 389 are also identical (pattern 23). Other *T. evansi* stocks included in this group are BAKIT 384, 390, 388, 429, 387, 402, 394 and 393.

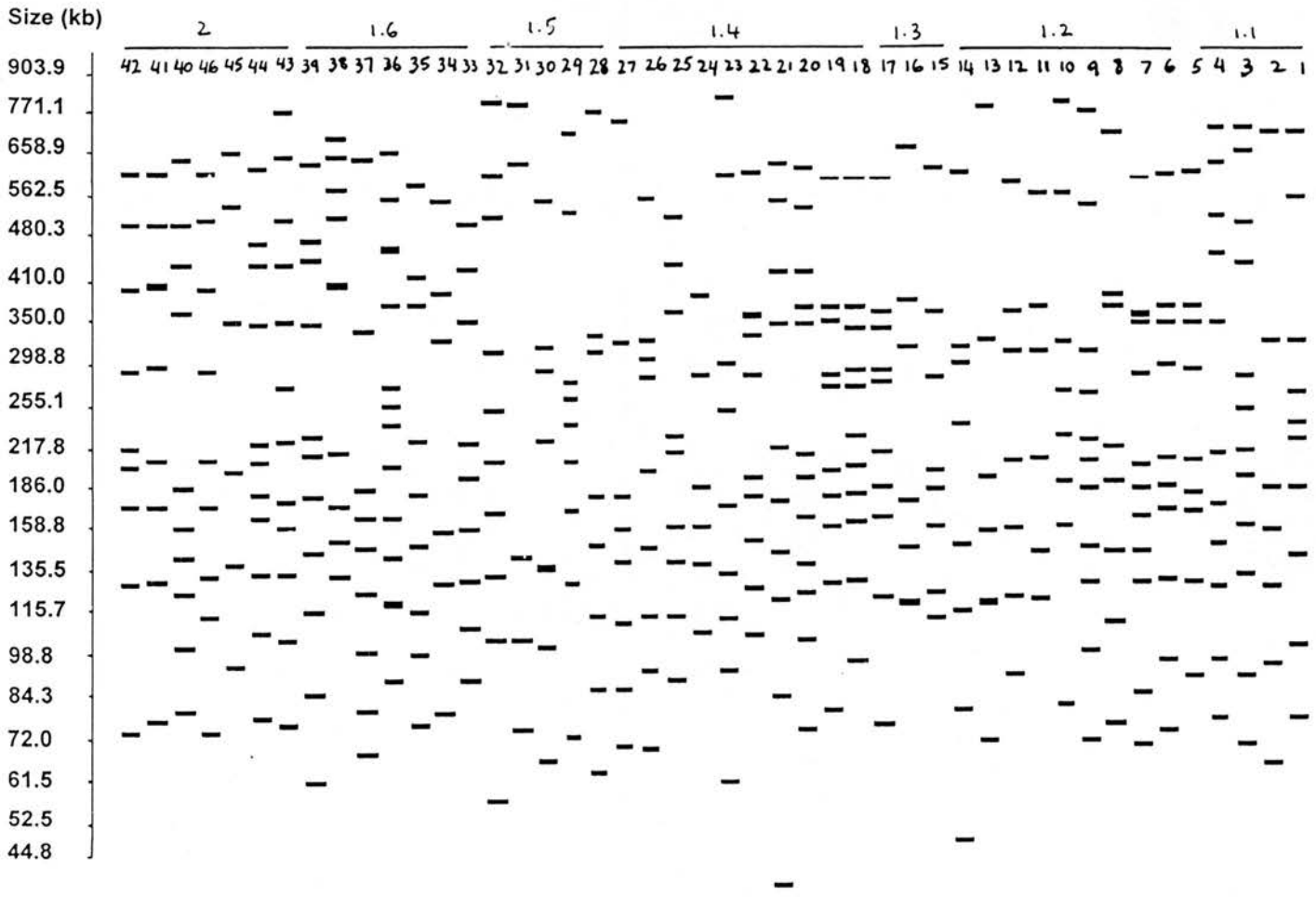


Figure 4.8. Indonesian karyotype banding patterns

Karyotype group 1.5 has 5 to 10 bands located between 60 to 950 kb size range and consist of 8 stocks belonging to pattern 28 to 32. Identical patterns were observed in BAKIT 422 and 423 from North Sumatra (pattern 28) and BAKIT 467, 475 and 496 from East Java (pattern 31). Other stocks belonging to karyotype group 1.5 include BAKIT 510/Central Java (pattern 29), 312/Aceh (pattern 30) and 482/East Java (pattern 32).

The karyotype group 1.6 consists of 7 stocks belonging to 7 patterns (pattern 33-39) and has 6 to 12 chromosomal bands between the size range of 70 to 950 kb. The stocks belonging to this group consist of BAKIT 381/North Sumatra/suramin resistant, 431/Lampung, 400/North Sumatra, 392/North Sumatra, 409/Lampung, 296/South Sulawesi and 461/South Kalimantan.

The stocks in karyotype group 2 have 6 to 10 chromosomal bands in the size range of 80 to 900 kb consisting of 10 stocks with 7 different patterns (pattern 40-46). Identical banding patterns was observed in stocks from Central Java (BAKIT 372) and North Sumatra (BAKIT 424, 427, 425), which belonged to pattern 40. Three stocks from West Java (BAKIT 503/pattern 41, 504/pattern 42, 294/pattern 44, 500/pattern 45), one from North Sumatra (BAKIT 399/pattern 43) and one stock from East Java (BAKIT 126/pattern 46) also belonged to the karyotype group 2.

Results on the analysis of banding pattern similarity are shown in the dendrogram shown in **Figure 4.9**. The banding patterns shown by the 80 stocks were compared and assigned to one of the seven karyotype groups using a 10% grouping level. The characteristics of each group are shown in **Table 4.2**.

Table 4.2. Karyotype group characteristics of the 80 *T. evansi* stocks isolated from 10 regions of Indonesia.

Karyotype		Chromosomal band size (kb)			Total no.
Pattern	Group	mc	Ic	Lc	of bands
1-5	1.1	2-3	7-9	0	9-12
6-14	1.2	1-3	3-8	0-1	6-11
15-17	1.3	1-2	5-9	0	6-11
18-27	1.4	1-4	4-9	0-1	8-12
28-32	1.5	2-3	2-7	0-1	5-10
33-39	1.6	1-4	4-10	0-1	6-12
40-46	2	1-3	5-8	0	6-10

mc = Minichromosomes (45-150 kb).
Ic = Intermediate chromosomes (150-900 kb).
Lc = Large chromosomes (900-1,050 kb).

FIGURE: 4.9

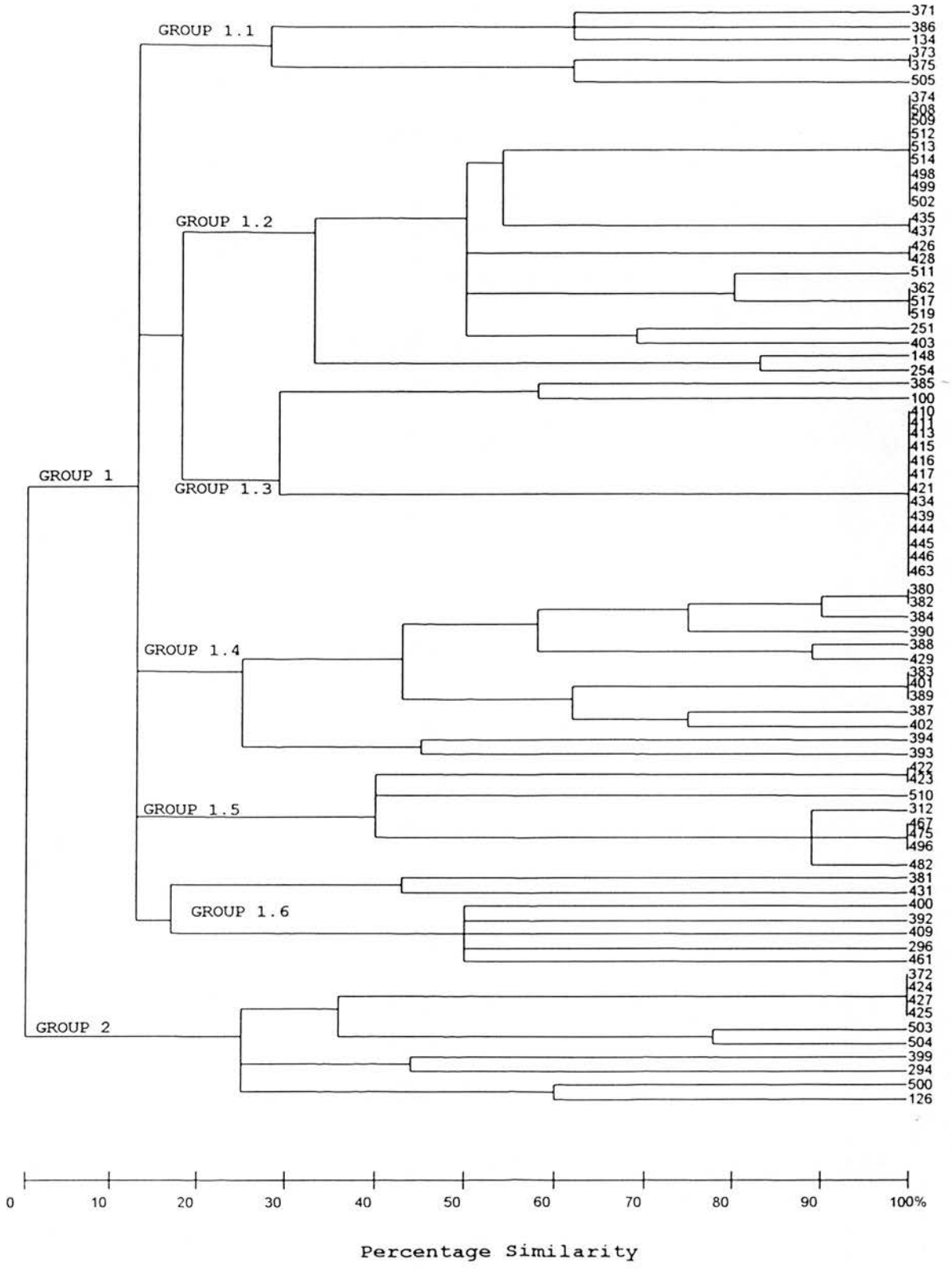


Figure 4.9. A dendrogram analysis of karyotype patterns of the 80 *T. evansi* stocks collected from 10 regions of Indonesia.

The karyotype patterns distributions of *T. evansi* stocks in Indonesia are shown in **Figure 4.10**. The map was drawn based on the presence of the karyotype groups in 10 regions in Indonesia. The numbers of stocks belonging to each karyotype group present in each of the 10 study areas in Indonesia are shown in **Table 4.3**.

Table 4.3. Karyotype group distribution of *T. evansi* stocks in 10 regions of Indonesia showing the number of stocks belonged to each karyotype group in each study area.

Isolation locality	Karyotype group (no. of stocks)							Total no. of stocks
	1.1	1.2	1.3	1.4	1.5	1.6	2	
Aceh	0	0	0	0	1	0	0	1
North Sumatra	1	2	1	13	2	3	4	26
Lampung	0	2	13	0	0	2	0	17
West Java	2	3	0	0	0	0	4	9
Central Java	3	7	0	0	1	0	1	12
East Java	0	0	0	0	4	0	1	5
Madura	0	0	3	0	0	0	0	3
North Sulawesi	0	2	1	0	0	0	0	3
South Sulawesi	0	1	0	0	0	1	0	2
South Kalimantan	0	1	0	0	0	1	0	2
Total	6	18	18	13	8	7	10	80

Karyotype group 1.2 is predominant in Indonesia and present in most of the study areas except East Java, Madura and Aceh, while karyotype group 1.4 was only found in *T. evansi* stocks collected from North Sumatra (13 stocks). North Sumatra has the most karyotype variations with all of the karyotype groups present in the area. Java has less karyotype group variations than North Sumatra with the absence of karyotype group 1.3; 1.4 and 1.6. Three karyotype groups were present in Lampung, karyotype group 1.2; 1.3 and 1.6. *Trypanosoma evansi* stocks from Aceh and Madura were represented by one karyotype group, 1.5 and 1.3 respectively. Two karyotype groups, 1.2 and 1.6, present in *T. evansi* stocks from South Kalimantan and South Sulawesi.

Karyotype pattern stability was noted in the stocks isolated from West and Central Java during the period of 6 years (1988 to 1994). It was noted that 9 out of 12 *T. evansi* stocks collected from West and Central Java had identical chromosomal banding patterns, which belong to the karyotype sub group 1.2.

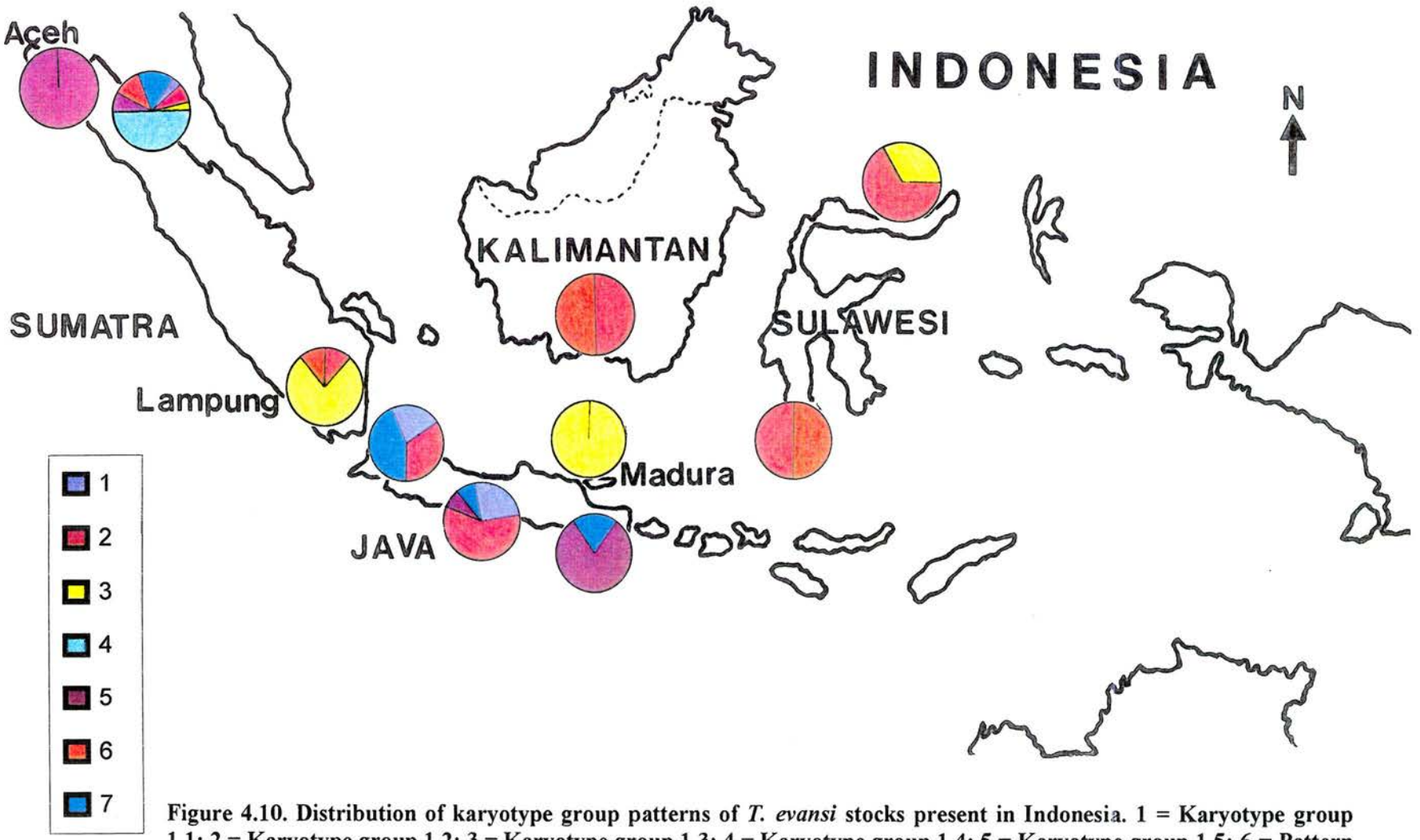


Figure 4.10. Distribution of karyotype group patterns of *T. evansi* stocks present in Indonesia. 1 = Karyotype group 1.1; 2 = Karyotype group 1.2; 3 = Karyotype group 1.3; 4 = Karyotype group 1.4; 5 = Karyotype group 1.5; 6 = Pattern I.6; 7 = Pattern 2.

4.3.3.2. Analysis of Karyotype Polymorphisms in T. evansi *Stocks Isolated from Transported Buffalo*

The chromosomal banding patterns shown by the stocks isolated in Central Java and North Sumatra were variable, although some similarities in the banding patterns were also noted, mostly in stocks collected at the same time. The chromosomal banding pattern analysis compared the karyotypes of trypanosomes isolated from: a) buffaloes from Central Java before and 13 months after transportation to North Sumatra; b) isolates from a) compared with trypanosome stocks isolated from local (North Sumatra) buffaloes 13, 24 and 32 months after the Central Java buffaloes were introduced.

a) Karyotypes of *T. evansi* isolated from Central Java buffaloes before and 13 months after transportation

Five stocks were collected before transportation, two of them (BAKIT 373 and BAKIT 375) showing identical chromosomal banding patterns (**Figure 4.11**). Seven to 12 chromosomal bands, between the size range of 74 to 900 kb, present in the Central Java stocks (**Figure 4.11**). The three stocks were grouped as karyotype group 1.1 (BAKIT 371, 373 and 375); one group 1.2 (BAKIT 374) and one group 2 (BAKIT 372) (**Table 4.4**).

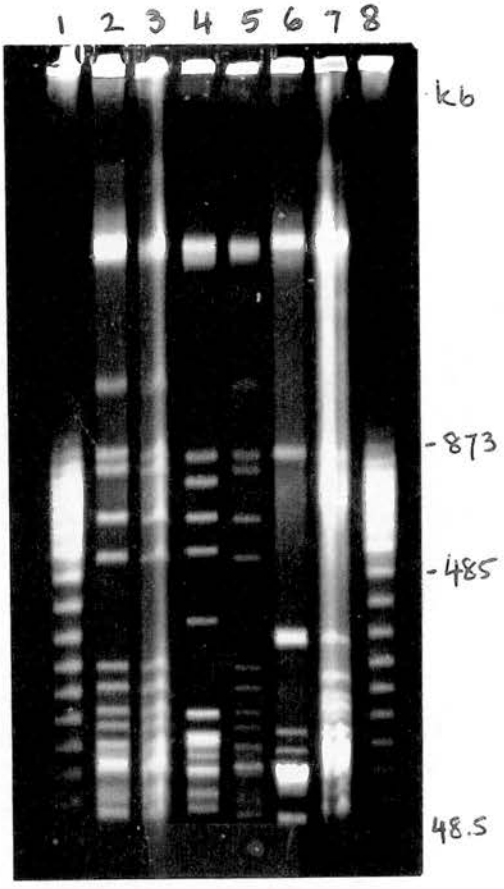
Thirteen months after the arrival of Central Java buffaloes in North Sumatra, eight *T. evansi* stocks were collected. However none of them were isolated from the same animals sampled before exportation. Post export patterns were 5 stocks (BAKIT 382, 384, 387, 383, 393) belonging to karyotype group 1.4; one group 1.1 (BAKIT 386) and two group 1.6 (BAKIT 381 and 392).

Table 4.4. Karyotype group present in *T. evansi* stocks isolated from buffaloes originated from Central Java and North Sumatra (local) before and after transportation (13, 24 and 32 months).

	Karyotype group (No. of stocks)							Total no.
	1.1	1.2	1.3	1.4	1.5	1.6	2	of stocks
Before transport.	3	1	0	0	0	0	1	5
After transport.:								
C. Java buffaloes	1	0	0	5	0	2	0	8
Local buffaloes:								
13 Months	0	0	1	5	0	0	0	6
24 Months	0	0	0	1	0	1	1	3
32 Months	0	2	0	1	2	0	3	8
Total	4	3	1	12	2	3	5	30

Figure 4.11. *Trypanosoma evansi* stocks isolated from buffaloes in Central Java before transportation to North Sumatra separated by Transverse Alternating Field Electrophoresis using conditions optimised to resolve DNA in the 50-900 kb size range.

Track 1, 8: Lambda DNA concatemer size standard
Track 2, 5: BAKIT 373 (Karyotype group 1.1)
Track 3: BAKIT 375 (Karyotype group 1.1)
Track 4: BAKIT 374 (Karyotype group 1.2)
Track 6: BAKIT 372 (Karyotype group 2)
Track 7: BAKIT 371 (Karyotype group 1.1)


1 2 3 4 5 6 7 8
kb
-873
-485
48.5

b) Karyotypes of *T. evansi* isolated from Central Java buffaloes before and from local buffaloes 13, 24 and 32 months after transportation

Analysis of the molecular karyotypes was also extended to examine the karyotypes of *T. evansi* stocks isolated from local buffaloes after the arrival of the Central Java buffaloes.

Six *T. evansi* stocks were collected from local buffaloes in North Sumatra, in addition to the 8 stocks isolated from the Central Java buffaloes 13 months after transportation. The karyotype patterns of *T. evansi* stocks isolated from Central Java and local buffaloes 13 months after transportation are shown in **Figure 4.12**. Identical karyotype patterns were noted in the stocks isolated from local and Central Java buffaloes. Two identical karyotypes were observed in BAKIT 380 (isolated from a local buffalo) and BAKIT 382 (isolated from a Central Java buffalo), both belong to karyotype group 1.4. BAKIT 383 (isolated from a Central Java buffalo) has an identical karyotype with that of BAKIT 389 (isolated from a local buffalo); both stocks also belonged to karyotype group 1.4.

The karyotype patterns of *T. evansi* stocks isolated from local buffaloes 13, 24 and 32 months after the Central Java buffaloes were transported are shown in **Figure 4.13**.

Three identical karyotypes were observed in 6 stocks collected at the third visit: between BAKIT 422 and 423 (group 1.5); BAKIT 424, 425 and 427 (group 2) and between BAKIT 426 and 428 (group 1.2).

Further analysis showed that one stock (BAKIT 372) isolated from a Central Java buffalo before transportation had an identical banding pattern to three stocks (BAKIT 424, 425 and 427) isolated from local buffaloes 32 months after the Central Java buffaloes were transported.

Figure 4.12. *Trypanosoma evansi* stocks separated by Transverse alternating field electrophoresis using conditions optimised to resolve DNA in the 50-900 kb size range. The *T. evansi* stocks were isolated from transported and local buffaloes 13 months after transportation unless stated otherwise.

Tracks 1, 10, 11, 20: Lambda DNA concatemer size standard
Track 2: BAKIT 385 (local; group 1.3)
Track 3: BAKIT 393 (C. Java; group 1.4)
Track 4: BAKIT 392 (C. Java; group1.6)
Track 5: BAKIT 388 (local; group 1.4)
Track 6: BAKIT 382 (C. Java; group 1.4)
Track 7: BAKIT 399 (local; collected after 24 months; group 2)
Track 8: BAKIT 390 (local; group 1.4)
Track 9: BAKIT 380 (local; group 1.4)
Track12: BAKIT 425 (local; collected after 32 months; group 2)
Track13: BAKIT 394 (local; group 1.4)
Track14: BAKIT 400 (local; collected after 24 months; group 1.6)
Track15: BAKIT 389 (C. Java; group 1.4)
Track16: BAKIT 387 (C. Java; group 1.4)
Track17: BAKIT 383 (C. Java; group 1.4)
Track18: BAKIT 381 (C. Java; group 1.6)
Track19: BAKIT 386 (C. Java; group 1.1).

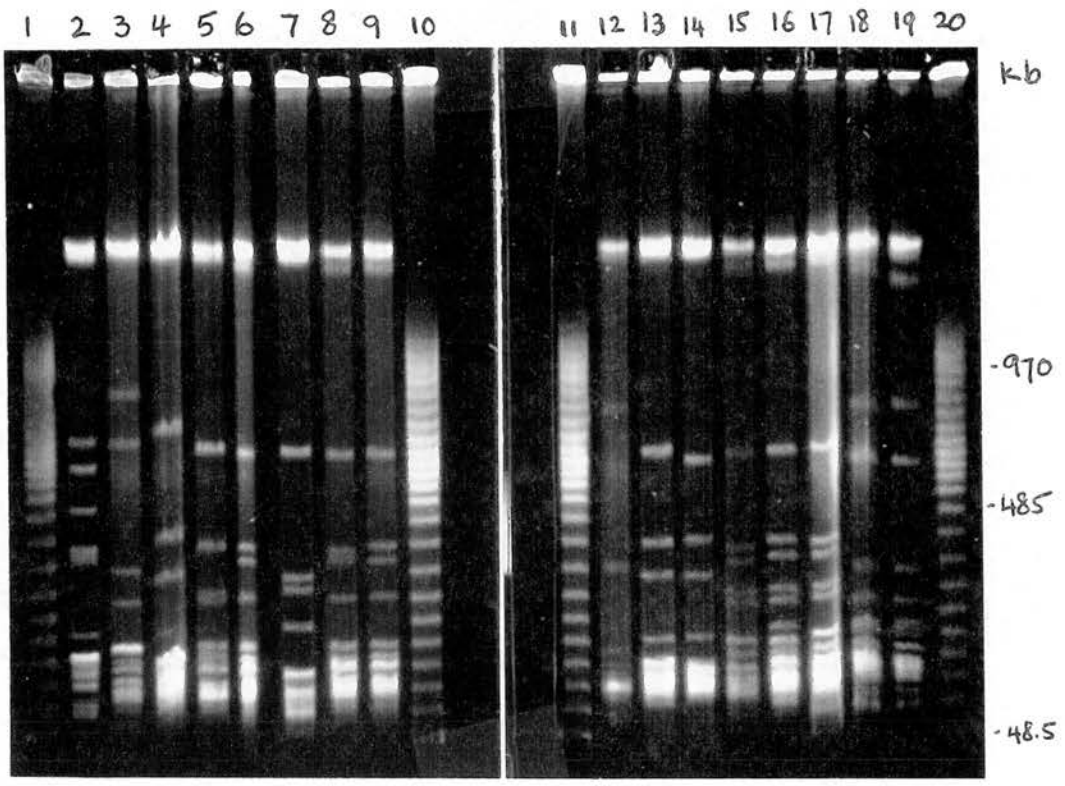
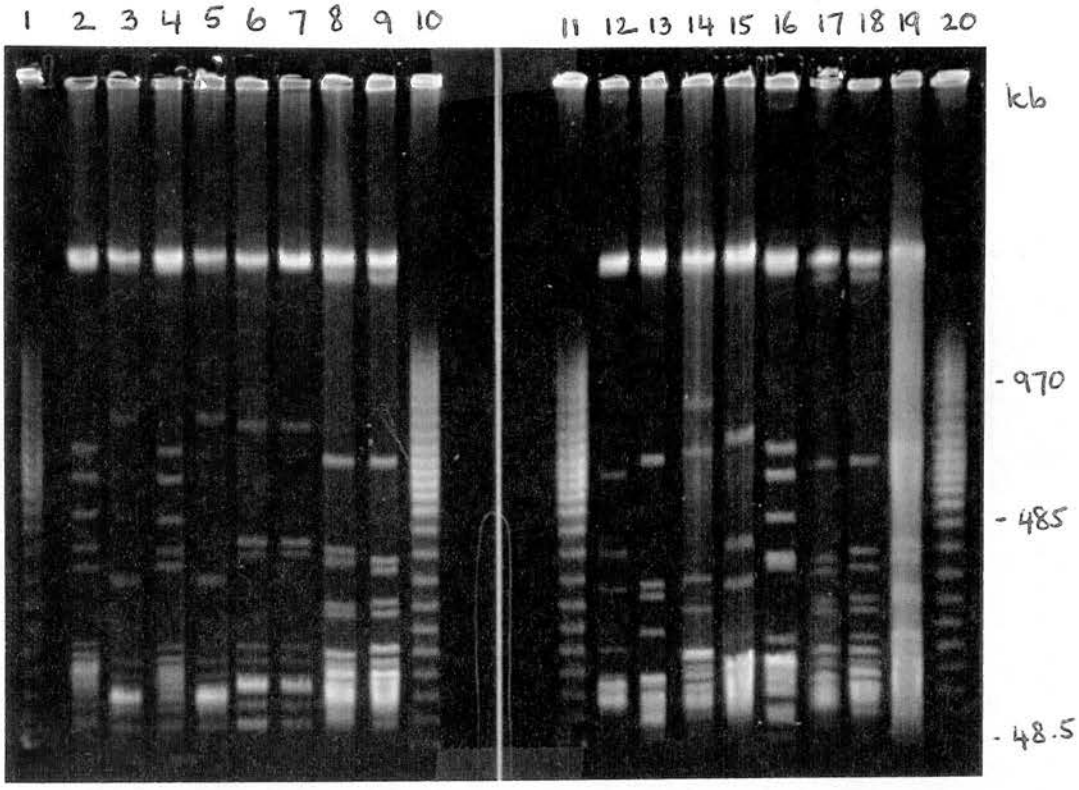

1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
kb
-970
-485
-48.5

Figure 4.13. *Trypanosoma evansi* stocks separated by Transverse alternating field electrophoresis using conditions optimised to resolve DNA in the 50-900 kb size range. The *T. evansi* stocks were isolated from local buffaloes (unless stated otherwise) in North Sumatra 13, 24 and 32 months after transportation.

Tracks 1, 10, 11, 20: Lambda DNA concatemer size standard
Track 2: BAKIT 428 (32 months; group 1.2)
Track 3: BAKIT 427 (32 months; group 2)
Track 4: BAKIT 426 (32 months; group1.2)
Track 5: BAKIT 424 (32 months; group 2)
Track 6: BAKIT 423 (32 months; group 1.5)
Track 7: BAKIT 422 (32 months; group 1.5)
Track 8: BAKIT 402 (24 months; group 1.4)
Track 9: BAKIT 401 (C.Java; 24 months; group 1.4)
Track12: BAKIT 400 (24 months; group 1.6)
Track13: BAKIT 399 (24 months; group 2)
Track14: BAKIT 393 (C. Java; 13 months; group 1.4)
Track15: BAKIT 392 (C. Java; 13 months; group 1.6)
Track16: BAKIT 385 (13 months; group 1.3)
Track17: BAKIT 389 (13 months; group 1.4)
Track18: BAKIT 387 (C. Java; 13 months; group 1.4)
Track19: BAKIT 383 (C. Java; 13 months; group 1.4)


1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
kb
- 970
- 485
- 48.5

The karyotype group variations of the stocks isolated from Central Java before and 13 months after transportation and *T. evansi* stocks isolated from local buffaloes 13 months after the Central Java buffaloes were introduced are shown in **Table 4.4**. The overall distribution of the karyotype groups of *T. evansi* isolated from transported buffaloes is illustrated in a diagram shown in **Figure 4.14.** Before transportation the karyotype group 1.1 was predominant in the *T. evansi* stocks isolated in Central Java. After 13 months in North Sumatra, the karyotype group 1.4 was predominant in the stocks isolated from Central Java buffaloes. Karyotype group 1.4 was also predominant in the stocks isolated from local (North Sumatra) buffaloes collected 13 months after the Central Java buffaloes were introduced.

Three stocks were collected from local buffaloes after 24 months, which belonged to karyotype group 1.4 (BAKIT 402); 1.6 (BAKIT 400) and 2 (BAKIT 399). Eight trypanosome stocks collected after 32 months from the local buffaloes belonged to karyotype group 1.2 (BAKIT 426, 428); 1.4 (BAKIT 429); 1.5 (BAKIT 422, 423) and 2 (BAKIT 424, 427, 425).

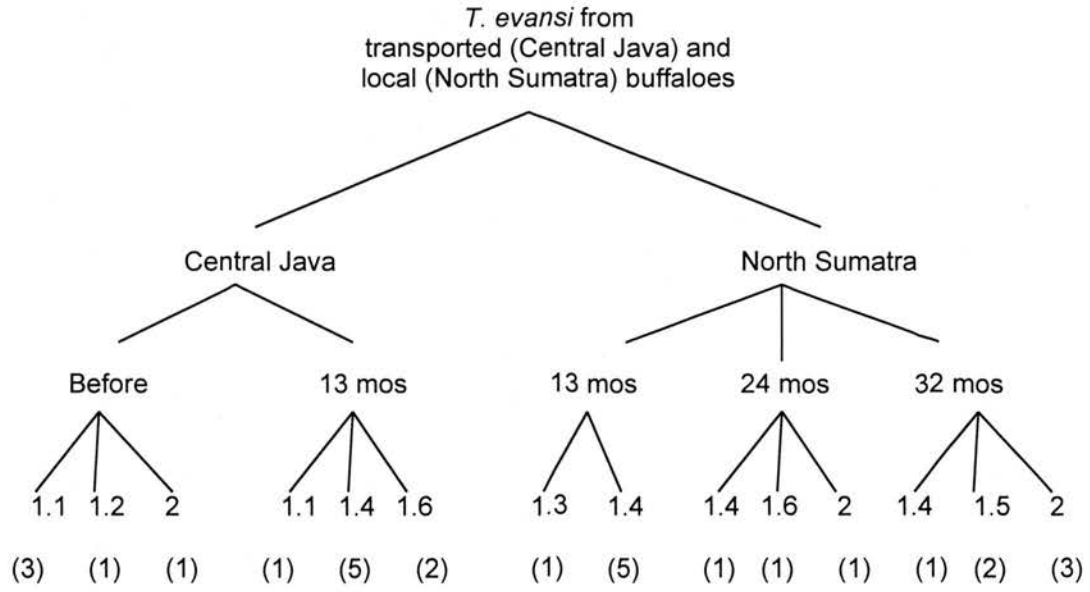


Figure 4.14. Diagram of the overall karyotype group distribution of *T. evansi* stocks isolated from transported buffaloes before and after transportation.

4.3.3.3. Analysis of Karyotype Patterns of T. evansi *Stocks Isolated from a Bali Cattle Feedlot in Lampung*

Very limited heterogeneity in the karyotype patterns was found in *T. evansi* stocks isolated from Bali cattle kept in a feedlot in Lampung (**Figure 4.15**). Identical chromosome banding patterns were seen in 13 out of 17 stocks collected during the study period, which belonged to pattern 17 in karyotype group 1.3.

Karyotype analysis detected three different karyotype groups in the 17 stocks isolated in Lampung. The karyotype groups present in the Lampung feedlot collected during the 5-months period of study are shown in **Table 4.5**. Eight *T. evansi* stocks were collected at the first visit; 7 of them belonging to karyotype group 1.3 (BAKIT 410, 411, 413, 415, 416, 417 and 421) and one (BAKIT 409) to group 1.6. Another set of 8 stocks were isolated at the second visit with 5 stocks belonging to karyotype group 1.3 (BAKIT 434, 439, 444, 445, 446), 2 stocks belonging to group 1.2 (BAKIT 435 and 437) and one (BAKIT 431) to group 1.6. At the last visit, because the animals had been treated with suramin, only one stock (BAKIT 463) which belonged to group 1.3 was collected.

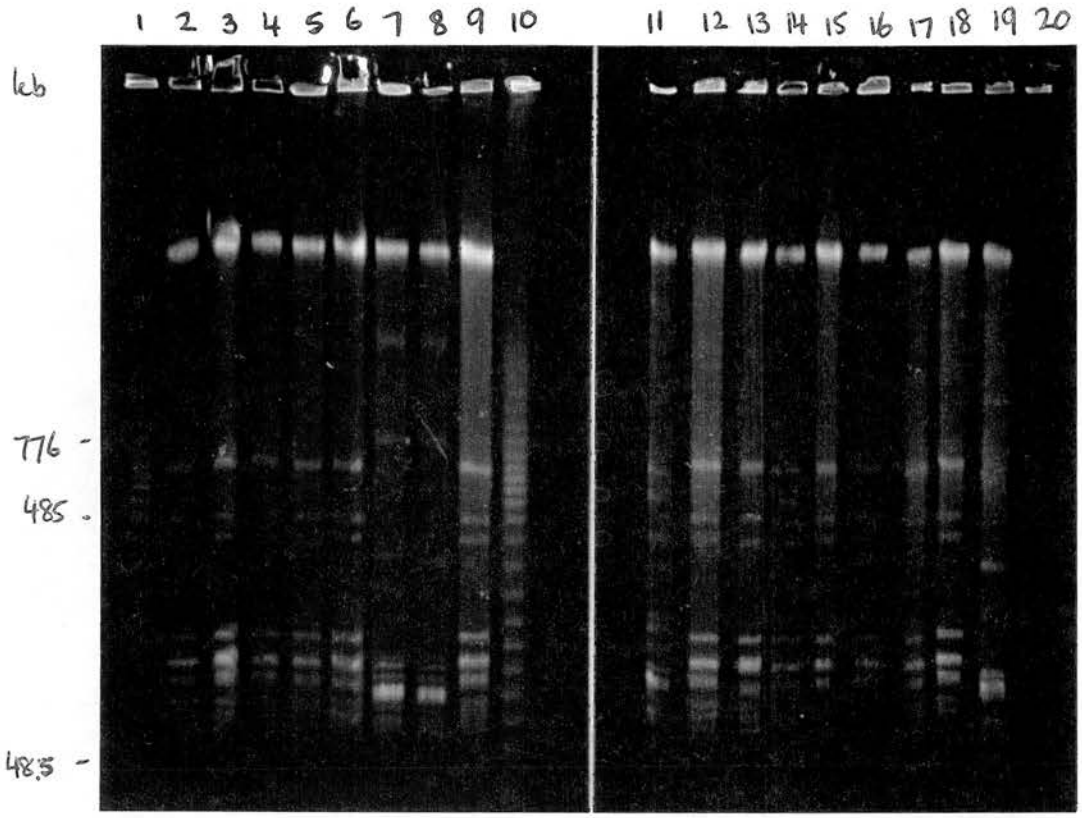
Two *T. evansi* stocks BAKIT 411 (group 1.3) and BAKIT 435 (group 1.2) were collected at one month interval (visits 1 and 2) from the same animal.

Table 4.5. Karyotype group of *T. evansi* stocks isolated from a group of Bali cattle feedlot in Lampung.

Visit	Host origination	Karyotype group (No. of stocks)							Total no. of stocks
		1.1	1.2	1.3	1.4	1.5	1.6	2	
I	Lampung	0	0	3	0	0	1	0	4
I	South Sumatra	0	0	4	0	0	0	0	4
II	Lampung	0	1	3	0	0	1	0	5
II	South Sumatra	0	1	2	0	0	0	0	3
III	Lampung	0	0	1	0	0	0	0	1
Total		0	2	13	0	0	2	0	17

Figure 4.15. *Trypanosoma evansi* stocks separated by Transverse alternating field electrophoresis using conditions optimised to resolve DNA in the 50-900 kb size range. The *T. evansi* stocks were isolated from a Bali cattle feed lot in Lampung.

Tracks 1, 10, 20: Lambda DNA concatemer size standard
Track 2: BAKIT 463 (Visit 3; group 1.3)
Track 3: BAKIT 446 (Visit 2; group 1.3)
Track 4: BAKIT 445 (Visit 2; group 1.3)
Track 5: BAKIT 444 (Visit 2; group 1.3)
Track 6: BAKIT 439 (Visit 2; group 1.3)
Track 7: BAKIT 437 (Visit 2; group 1.2)
Track 8: BAKIT 435 (Visit 2; group 1.2)
Track 9: BAKIT 434 (Visit 2; group 1.3)
Track11: BAKIT 431 (Visit 2; group 1.6)
Track12: BAKIT 421 (Visit 1; group 1.3)
Track13: BAKIT 417 (Visit 1; group 1.3)
Track14: BAKIT 416 (Visit 1; group 1.3)
Track15: BAKIT 415 (Visit 1; group 1.3)
Track16: BAKIT 413 (Visit 1; group 1.3)
Track17: BAKIT 411 (Visit 1; group 1.3)
Track18: BAKIT 410 (Visit 1; group 1.3)
Track19: BAKIT 409 (Visit 1; group 1.6)


1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
lcb
776
485
48.5

4.3.3.4. Comparison of the Karyotype Patterns between T. evansi *with* T. brucei *and* T. congolense

Figure 4.16 showed the variation in the karyotype patterns of the 14 representative *T. evansi* stocks and the chromosome profiles of *T. brucei* and *T. congolense* (**Table 4.6**). The analysis of the banding patterns was expanded to compare the band sizes in a wider size range, between 50 kb to 2,200 kb.

Table 4.6. *Trypanosoma evansi* stocks used for karyotype comparison with *T. brucei* and *T. congolense*.

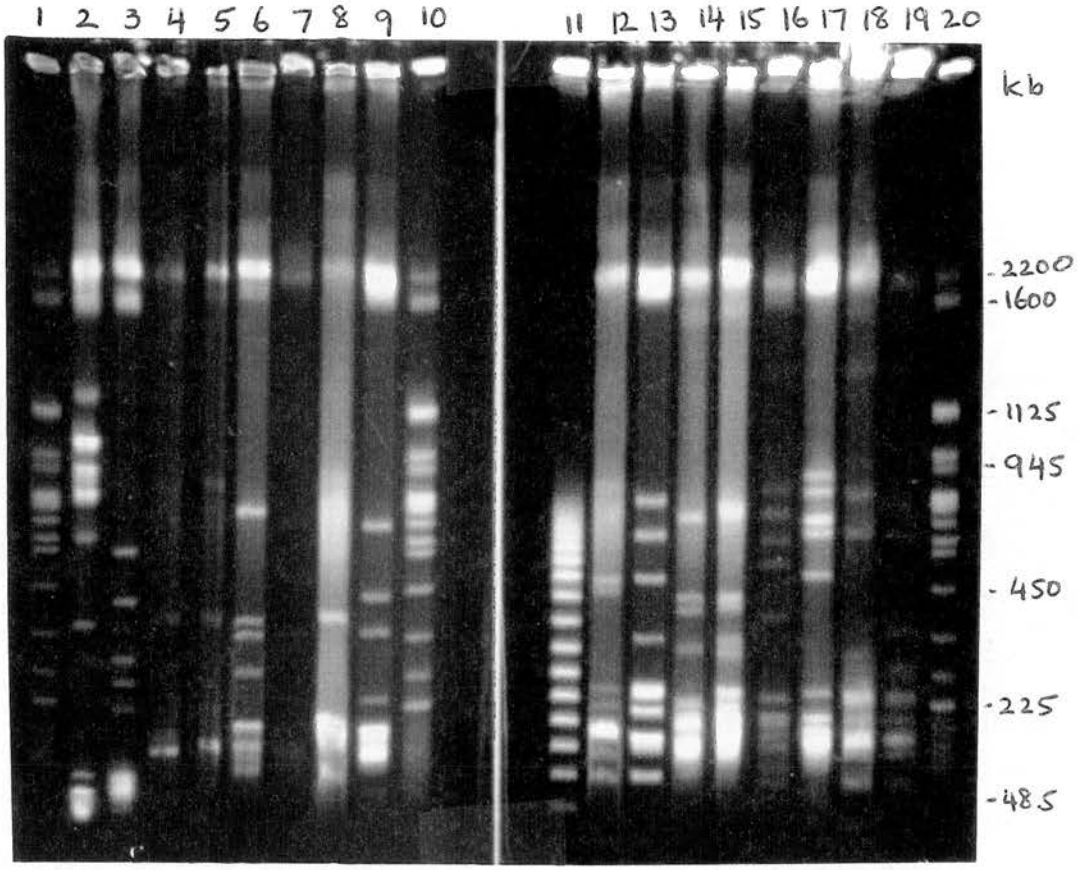
Trypanosome	Stock No	Isolation locality	Karyotype pattern	Karyotype group
T. brucei	TREU 2177	Uganda		
T. congolense	TREU 2193	Nigeria		
T. evansi	BAKIT 503	Central Java	41	2
T. evansi	BAKIT 427	North Sumatra	40	2
T. evansi	BAKIT 399	Central Java	43	2
T. evansi	BAKIT 500	West Java	45	2
T. evansi	BAKIT 409	Lampung	37	1.6
T. evansi	BAKIT 461	South Kalimantan	39	1.6
T. evansi	BAKIT 423	North Sumatra	28	1.5
T. evansi	BAKIT 467	East Java	31	1.5
T. evansi	BAKIT 382	North Sumatra	18	1.4
T. evansi	BAKIT 401	North Sumatra	23	1.4
T. evansi	BAKIT 508	Central Java	6	1.2
T. evansi	BAKIT 100	North Sulawesi	16	1.3
T. evansi	BAKIT 505	West Java	5	1.1
T. evansi	BAKIT 134	West Java	3	1.1

The chromosomal banding pattern of *T. brucei* (TREU 2177/Uganda) showed 7 resolved chromosomal bands in the size range of 200-2392 kb and a smear of bands smaller than 80 kb. *Trypanosoma congolense* (TREU 2193/Nigeria) has 9 chromosomal bands in the size range of 50 to 2244 kb and a smear of the chromosomes smaller than 50 kb. The optimised running condition for PFGE applied does not separate chromosomal bands of smaller than 50 kb, however, it was estimated that the smallest size in *T. congolense* was approximately 10 kb.

The main difference in the banding pattern between *T. congolense* and both *T. brucei* and *T. evansi* is in the size of the minichromosomes. The minichromosomes of the *T. congolense* stock studied are smaller than those of *T. evansi* and *T. brucei*. It was also noted that *T. congolense* has fewer chromosomal bands in the size range of 150 to 750 kb than both *T. evansi* and *T. brucei*.

Figure 4.16. Separation of *Trypanosoma congolense*, *T. brucei* and *T. evansi* stocks by Transverse alternating field electrophoresis using the optimised conditions to resolve DNA in the 50-900 kb size range.

Tracks 1, 10, 20: *Saccharomyces cerevisiae* size standard marker;
Track11: lambda DNA concatemer size standard
Track 2: TREU 2193/*T. congolense*
Track 3: TREU 2177/*T. brucei*
Track 4: BAKIT 503 (group 2)
Track 5: BAKIT 427 (group 2)
Track 6: BAKIT 399 (group 2)
Track 7: BAKIT 500 (group 2)
Track 8: BAKIT 409 (group 1.6)
Track 9: BAKIT 461 (group 1.6)
Track12: BAKIT 423 (group 1.5)
Track13: BAKIT 467 (group 1.5)
Track14: BAKIT 382 (group 1.4)
Track15: BAKIT 401 (group 1.4)
Track16: BAKIT 508 (group 1.2)
Track17: BAKIT 100 (group 1.3)
Track18: BAKIT 505 (group 1.1)
Track19: BAKIT 134 (group 1.1)


1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
kb
2200
1600
1125
945
450
225
485

The difference between the karyotype patterns of the *T. brucei* and *T. evansi* stocks studied, was the lack of intermediate sized chromosomal bands in *T. brucei* in the size range of 600-900 kb. The difference between *T. congolense* and *T. evansi* is the lack of chromosomal bands in the size range of 50-380 kb in *T. congolense*.

Table 4.7 summarises the chromosomal banding patterns of *T. evansi*, *T. brucei* and *T. congolense* stocks used in the study. As in *T. evansi*, the *T. brucei* (TREU 2177/ Uganda) karyotype studied can be divided into 4 size classes: a) very large chromosomes that remained in the gel slot; b) 2 chromosomal bands in the size range of 1.6-2.4 Mb and the chromosomes that remained in the compression zone; c) 5 intermediate sized chromosomes within the size range of 200 to 600 kb; d) a smear of within the size range of 45 to 80 kb.

The PFGE running conditions has separated the *T. congolense* karyotype into 4 size classes: a) very large chromosomes that remained in the gel slot; b) 2 chromosomal bands in the size range of 1.6-2.2 Mb and the chromosomes remained in the compression zone; c) 6 chromosomal bands in the size range of 400 kb to 1200 kb; d) a smear of small chromosomes of less than 50 kb.

Table 4.7. A summary of the chromosomal banding patterns of *T. evansi*, *T. brucei* and *T. congolense* stocks used in the study. All stocks have DNA that remained in the gel slot and unresolved bands in the compression zones.

	Lc	Ic	mc
T. evansi	1-2	2-10	1-4 or smear
T. brucei	2	5	Smear
T. congolense	2	6	Smear

Lc = Large chromosomes in the megabase size range (1.6-2.2 Mb in *T. evansi* and *T. congolense*; 1.6-2.4 Mb in *T. brucei*).

Ic = Intermediate sized chromosomes (150-900 kb in *T. evansi*; 200-600 kb in *T. brucei*; 400-1,200 kb in *T. congolense*).

mc = minichromosomes (45-150 kb in *T. evansi*; 45-80 kb in *T. brucei*; 10-80 kb in *T. congolense*)

4.3.4. DISCUSSION

4.3.4.1. Karyotype Variability in T. evansi *Stocks in Indonesia*

A high degree of variability in the karyotype patterns was shown by PFGE which was in marked contrast to the limited heterogeneity reported for *T. evansi* stocks in Kenya (Waitumbi and Young, 1994) and China (Lun *et al.*, 1992b). The differences in the degree of karyotype variability between *T. evansi* stocks from Indonesia and those from Kenya and China might be due to several factors. Differences in the techniques applied and the chromosomal band size range examined might be one of the reasons for more differences in karyotype variability observed in *T. evansi* stocks from Indonesia than those from Kenya and China. This study used a transverse alternating field electrophoresis (TAFE) system resulting in compressed highly resolved banding patterns in straight lanes. This result is obtained by the positioning of the electrodes on both of the gel surfaces producing straight lanes, and the changes in the electric fields down the gel produces compressed, highly resolved bands from the top to the bottom of the gel (Beckman Inst. Manual). The band sizes of *T. evansi* stocks separated by the TAFE system were determined by reciprocal fit of the distance travelled by the Lambda DNA size marker fragments using a whole band analyser (Millipore, USA) which estimates the bands sizes between 50-900 kb size range accurately.

Other factors contributing to the apparent chromosomal size variability in *T. evansi* might be caused by chromosomal rearrangements (Van der Ploeg *et al.*, 1984b), which involves telomere growth and contraction as described in *T. brucei* (Bernards *et al.*, 1983). The activation of VSG gene expression, associated with antigenic variation, involved transposition of hundreds of kilobase pairs which caused the movement of telomeric VSG genes from one chromosome to the other. This resulted in marked differences in the chromosomal banding patterns (between 50 kb to 2 Mb) in different variants of *T. brucei* (Van der Ploeg *et al.*, 1984b; Shea *et al.*, 1986). It was, therefore, suggested (Lun *et al.*, 1992) that identical karyotypes indicated close relatedness among the stocks analysed.

Karyotype variability in *T. brucei* was apparent as a result of genetic exchange during the sexual stage in the tsetse fly vector (Jenni *et al.*, 1986). The alteration in the chromosomal size due to the occurrence of genetic exchange had been reported in *T. brucei* (Wells *et al.*, 1987; Gibson and Garside, 1991; Gibson *et al.*, 1992; Gibson and Whittington, 1993; Hide, Weburn, Tait *et al.*, 1994; Schweizer, Posphical, Hide *et al.*, 1994; Gibson *et al.*, 1995; Tait, Buchanan, Hide *et al.*, 1996), in *T. cruzi* (Bogliolo, Laurapires and Gibson, 1996) and in *Leishmania* (Pages *et al.*, 1989). However, the presence of a sexual stage has not been described in *T. evansi*.

The extensive karyotype variability in *T. evansi* might also relate to the variations in the DNA content among different strains. However there is a lack of information on the estimated DNA content in *T. evansi*, except the one reported by Baker (1961). The karyotype variability due to the variation in the DNA content among different isolates and among clonal isolates derived from a single clone had been described in *T. cruzi* (Henriksson *et al.*, 1996a).

In addition to the factors mentioned above, the high degree of karyotypes variability in Indonesia might be correlated with the geographic factor and historically different origination of *T. evansi* strains introduced. The history of the introduction of surra to the Indonesian archipelago is different from those of Kenya and China. Hoare (1972) postulated that *T. evansi* evolved from *T. brucei* by adaptation to mechanical means of transmission when caravans of camels came into contact with *T. brucei* and then returned to tsetse-free areas. Based on the marked differences in the karyotype groups, between stocks isolated from the north-west and south of China, Lun *et al.* (1992b) suggested the multiple introduction of *T. evansi* via the camel trade to north-west China in addition to the introduction of infected mules from Burma (Luckins, 1988). In Indonesia *T. evansi* might have entered by transmission of the parasite from transported animals from India (Dieleman, 1983) at the beginning of the 20th century, when India was heavily infested with Surra (Hoare, 1972). However, the introduction of *T. evansi* to Indonesia might have been started a long time before it was first reported by Penning in 1903.

Livestock might have been brought into the country since the establishment of an agricultural system. A dominant agrarian state has been established since the 8th century in Central Java and in the 14th century the Majapahit Empire accomplished the synthesis between maritime state and agrarian state in Indonesia. In 1446 Islam was established in Malaka; at the same time the Indian Muslim traders entered Indonesia. In 1505 the Portuguese started their search for spices through the Cape of Hope to Ormuz, India, Ceylon, Malaka, Indonesia which was then established in Maluku, the spice islands. In 1641 the Dutch took over the spice bases from the Portuguese and established the spice route: Amsterdam, Cape Town, Mauritius, Persia, India, Ceylon, Malaka, Indonesia and ended in the Maluku islands.

Livestock might have been brought in to Indonesia by the Indians (15th century), Portuguese (16th century) or Dutch (17th century); for example horses for their cavalry, cattle for their trade. Some of these animals might have been infected during the stopovers and suffered from subclinical *T. evansi* infections, which eventually transmitted among local animals. This multiple introduction of *T. evansi*, presumably consisting of mixed karyotypes, might cause the high degree of variability in *T. evansi* stocks. *Trypanosoma evansi* stocks of different karyotypes introduced were then isolated in islands in the Indonesian archipelago. Transportation of livestock suffering from subclinical infection with *T. evansi* between

endemic areas had caused multiple introductions of different *T. evansi* strains. In spite of the quarantine regimens applied, the widespread presence of the Tabanid flies, the potential vector for surra in Indonesia, is significant. This condition facilitates the *T. evansi* transmission among transported and local animals.

It is known that *T. evansi* originated from *T. brucei* (Hoare, 1972), which has different karyotypes, however, in Kenya and China *T. evansi* eventually evolved to a limited number of karyotypes. In Indonesia, although an extremely high degree of molecular karyotype differences were detected, some degree of karyotype stability was also observed. Molecular karyotype stability had been exemplified in the indigenous stocks collected from restricted areas of Java between the period of 1989 to 1994. The mechanism of the presence of karyotype stability is not known, however, it has been suggested that homogeneity in the karyotype patterns present in different areas occurs where trypanocidal regimens are applied regularly (Waitumbi *et al.*, 1994). This does not appear to be the reason in Java, as the chemotherapy control is not applied regularly due to the high price and difficulties in administration of the drug (Naganol®, Bayer). The other trypanocidal drug available in Indonesia, Isometamidium chloride (Trypamidium®, Specia), is not effective against *T. evansi* infections in Indonesia (Sukanto *et al.*, 1990). The karyotype pattern stability in Indonesia might be associated with the island isolation where *T. evansi* stocks might have adapted themselves while circulating in the restricted area.

Analysis of karyotype patterns has shown 3 large karyotype groups in stocks from West & Central Java (karyotype group 1.2); Lampung (karyotype group 1.3) and North Sumatra (karyotype group 1.4). The *T. evansi* stocks from West Java, in fact, were isolated from buffaloes transported from Central Java. These 3 karyotype group patterns which were identified will be referred to in future as: Central Java, Lampung and North Sumatra patterns respectively. This result indicated the presence of isolation locality of the *T. evansi* stocks studied.

The Central Java patterns are more closely related (based on the grouping level of ~20% similarities) to those of Lampung than to the North Sumatra stocks. This might indicate the close relationships of the stocks from geographically close isolation localities, in which Java is closer to Lampung than to North Sumatra. However, identical karyotype pattern in stocks collected from Lampung and Madura outbreaks indicated the presence of a single infection by one pattern.

Molecular characterisation based on PFGE has been successful in differentiating the chromosome profiles in 80 *T. evansi* stocks from Indonesia. This study has suggested that the chromosomal banding patterns of the *T. evansi* field stocks represent the karyotypes circulating in an area and showed the evidence of geographical isolation. All of the *T. evansi* field stocks used in the study were collected from infected livestock and subinoculated into

mice prior to stabilate preparation and subsequent agarose blocks preparations. Karyotypes *T. evansi* field stocks collected from Indonesia are stable. The agarose blocks were prepared from the field stocks without cloning; it was possible that the field stocks contain a mixture of several stocks. In a separate study it was shown that the karyotype pattern of cloned and uncloned stocks are identical, except in one population that consisted of a mixed infection. This suggested that the cloning of trypanosome field stocks for karyotype study is unnecessary. All of the stocks were run in the gel at least twice; all showed identical results under the same running conditions. Different preparation of the agarose blocks of the same stock showed identical karyotypes.

4.3.4.2. Karyotype Variability in T. evansi *Stocks Isolated from Transported Buffalo*

Previous analysis has shown that *T. evansi* stocks from Central Java predominantly belonged to karyotype group 1.1. However, after 13 months in North Sumatra, the stocks isolated from the same group of buffaloes predominantly belonged to karyotype group 1.4, which, by coincidence, was the predominant karyotype in the local buffaloes at the same time. These results indicated the presence of stock isolation in North Sumatra and the Central Java buffaloes might have had acquired infection with local stock during the 13 months of their establishment in North Sumatra. The karyotype group 1.4 was not detected anywhere else in Indonesia except in North Sumatra. However, it is not known whether this pattern is locally stable as samples were not taken in North Sumatra before the transportation of the buffaloes from Central Java.

One stock isolated from a Central Java buffalo has an identical karyotype pattern with three stocks isolated from local buffaloes 32 months after transportation. Despite treatment with suramin (Naganol®, Bayer), given to the buffaloes prior to the transportation, this result indicated the introduction of *T. evansi* stock from Central Java to North Sumatra. The introduction of the imported stock to local buffaloes might indicate the inefficient treatment application on buffaloes before transportation or might imply the presence of drug resistance.

This study has suggested the importance of taking preventative measures, before transporting animals between endemic areas, in order to reduce the risk of *T. evansi* transmission among the transported and local livestock to avoid infections with "new" strains with different pathogenicity.

4.3.4.3. Karyotype Variability in T. evansi *Stocks Collected from a Bali Cattle Feedlot in Lampung*

Identical karyotype patterns in *T. evansi* stocks isolated from animals kept in close proximity, such as in a feedlot, indicated a single infection among the animals with one karyotype pattern and eventually causing an outbreak in the area. The presence of flies, which play an important role in the disease transmission, was significant at the time of sampling in the Bali cattle feedlot in Lampung. The number of cases of surra was decreased significantly after the animals were treated with Suramin (Naganol®, Bayer) on the second visit, which indicated that Suramin is still the drug of choice for the treatment of surra in this area. It is not known whether the animals were infected when they were brought in, or they were infected when they came in to the area. It is also not known if the pattern is unique to the Lampung feedlot area as samples from areas outside the feedlot were not taken.

Two stocks collected from the same animal in the first and second visit in the Lampung feedlot showed a different karyotype pattern. The animal might have been infected with two different populations, with the second population appearing when the first population disappeared. It is also possible that the animals had been superinfected with a different strain.

The analysis on the karyotype patters of *T. evansi* stocks from Indonesia has shown that the chromosomal banding patterns are not highly conserved among the 80 stocks studied regarding the chromosome size and composition. Molecular karyotype detects intra-species differences which coincides with the stock isolation locality. The study has indicated that molecular karyotype using PFGE is useful for epidemiological studies and for stock identification of any new strain introduced in an area.

4.3.4.4. Comparison between T. evansi *Karyotypes with* T. brucei *and* T. congolense

The size classes division shown by *T. congolense* and *T. brucei* karyotype patterns are similar to those of *T. evansi.* This study has shown that *T. congolense* has less intermediate size chromosomal bands than those of *T. brucei* and *T. evansi,* and the sizes of the minichromosomes are smaller in *T. congolense* than in *T. brucei* and *T. evansi.* Results of this study were in concordance to that of Gibson and Borst (1986); who detected the small size (25 to 50 kb) of minichromosomes of *T. congolense.* However the minichromosomes sizes of *T. brucei* stock used in this study were slightly smaller (45 kb to 80 kb) than that studied by Gibson and Borst (1986). Gibson and Borst (1986) showed that the *T. evansi* stock studied hardly had minichromosomes in the size range of 50 to 150 kb; whereas *T.*

brucei had approximately 100 minichromosomes in that size range. This study detected 1-4 minichromosomal bands in the size range of 45-150 kb, however, some stocks showed a smear of minichromosomes. The PFGE optimised-running condition applied in this study did not resolve chromosomal bands in the small size range (smaller than 50 or 100 kb). In pulsed-field gel electrophoresis, when the pulse times are increased to resolve larger bands, the smaller molecules run as unresolved bands because the velocity was also converged at a longer pulse time (Birren *et al.*, 1988).

The chromosomes of *T. evansi* stocks showed most variable patterns between the size range of 150 kb to 1,050 kb. *Trypanosoma brucei* has intermediate sized chromosomes between 200 kb to 600 kb and *T. congolense* has between 400 kb to 1200 kb. Gibson and Borst (1986) applied a different PFGE system and reported that *T. evansi* has a dense chromosome band between 200-400 kb and *T. brucei* has 5 to 6 bands in the same area. Because of the optimised running condition applied in this study, to separate bands in the size range of 50 kb to 900 kb, the chromosomal bands of *T. evansi* were well resolved in this region. *Trypanosoma congolense* has more bands within the size range of 600-1200 kb compared to those of *T. evansi* and *T. brucei*. *Trypanosoma evansi*, however, has more bands between 100-400 kb size range than *T. congolense*. This shows that *T. congolense* has different chromosome organisation from *T. evansi* and *T. brucei*.

4.4. COMPARISON OF DRUG-SENSITIVE AND DRUG-RESISTANT *T. EVANSI* BY KARYOTYPE ANALYSIS

4.4.1. INTRODUCTION

Control of trypanosomiasis basically relies on chemotherapy of the infected animals since vector control strategies are not efficient and immunisation is not yet possible due to antigenic variation. The main constraint of using chemotherapeutic and chemoprophylactic agents is the induction of drug resistance. Drug resistance in the field has been assumed to occur because of the reappearance of parasitaemia after drug treatment. Superinfections with different strains following treatment might also occur. Prolonged and frequent use of trypanocidal drugs in areas of high challenge areas (Clausen, Sidibe, Kabore *et al.*, 1992) and subtheurapeutic dosage given to animals when the necessity of prophylactic treatment was not certain (Van Zwieten, 1932) have been the major contributors to drug resistance in trypanosomiasis. A limited number of drugs have been used extensively to combat trypanosomiasis and drug resistant strains of trypanosomes have developed. Evidence of the development of drug resistant *T. evansi* strains has been reported in Kenya (Gitatha, 1980; Schillinger, 1985); Sudan (Luckins *et al.*, 1979; Abebe, Jones and Boid, 1983; Boid, 1988); India (Gill, 1971); China (Zhang *et al.*, 1993) and Indonesia (Sukanto *et al.*, 1990).

It is therefore, important to identify and monitor the spread of drug resistant populations in order to anticipate the appropriate disease control strategy. Several methods have been developed to identify drug resistance among *T. evansi* stocks as discussed in Section 2.3.7. The present study was carried out to investigate whether or not there was any difference between the karyotypes of drug sensitive and drug resistant stocks, specifically for suramin and Cymelarsan.

4.4.2. MATERIALS AND METHODS

4.4.2.1. Trypanosomes

Two cloned, culture adapted *T. evansi* stocks, originally from Indonesia, were used in the study. The stock, TREU 1981 (originally BAKIT 148 isolated in North Sulawesi), which is sensitive to Cymelarsan was obtained from BALITVET, Indonesia; as a cryopreserved stabilate. TREU 1981was adapted to cell-free culture conditions resistant to cymelarsan induced to a level of >40 mg/kg (MIC) by Dr. C. A. Ross in CTVM. The culture adapted form of TREU 1840 (originally from Indonesia) was a gift from Dr. E. Zweygarth, KETRI, Kenya. Resistance to suramin was induced in this stock, to a level of 100 ng/ml (MIC), by Dr. D.V. Sutherland in CTVM.

Four field isolates of *T. evansi*, BAKIT 294, BAKIT 126, BAKIT 134 and BAKIT 381 previously tested for drug sensitivity against suramin, diminazene aceturate and isometamidium chloride by the method described by Rottcher and Heising (1981), were found to be resistant to suramin (Sukanto *et al.*, 1990). These 4 stocks were included in the analysis.

4.4.2.2. Preparation of Agarose Blocks

TREU 1840 and 1981, original drug sensitive population and resistant populations, were grown in 200 ml culture tubes by Dr. C. A. Ross and Dr. D.V. Sutherland. Trypanosomes were separated from the culture medium by centrifugation at 3,000 rpm for 20 minutes. The trypanosome pellets were then washed with PSG by centrifugation three times, for 20 minutes each wash, and the number of trypanosomes in each population was estimated. Two agarose blocks containing 2 million trypanosomes in each block was prepared according to the method described in Section 3.2.4.

4.4.2.3. Transverse Alternating Field Electrophoresis

The TAFE running conditions used for separating *T. evansi* chromosomes was described in Section 4.2.2.4.2, which separates chromosomal bands between 50-500 kb size range.

4.4.3. RESULTS

The TAFE conditions applied in this study clearly resolved 10 of the lambda DNA fragments (48.5-485 kb). The karyotype patterns of the drug resistant and drug sensitive stocks of *T. evansi* were shown in **Figure 4.17**. The field stocks, BAKIT 294; BAKIT 126; BAKIT 134 and BAKIT 381 were previously subjected to different electrophoresis conditions which separates bands between 50-900 kb size range (Section 4.3.2.2) and assigned as karyotype group 1.1 (BAKIT 134), 1.6 (BAKIT 381) and 2 (BAKIT 126 and 294).

In the present running conditions, the TAFE detected 5 to 15 chromosomal bands between sizes 50-500 kb, with variable karyotype patterns in the 4 *T. evansi* field stocks resistant to suramin. BAKIT 294 (lane 2, **Figure 4.17**) has 5 chromosomal bands in the size range of 70-194 kb, BAKIT 126 (lane 3, **Figure 4.17**) has 10 chromosomal bands between 90-388 kb size range. BAKIT 134 (lane 4, **Figure 4.17**) and BAKIT 381 (lane 5, **Figure 4.17**) had 14 and 15 chromosomal bands respectively distributed between 70-400 kb size range, these two stocks have similar banding patterns.

Differences in the chromosome profiles were not observed between suramin-resistant and suramin-sensitive stocks (**Figure 4.17**, lanes 6 and 7 respectively) and between cymelarsan-resistant and cymelarsan-sensitive stocks (**Figure 4.17**, lanes 8 and 9 respectively). The *Trypanosoma evansi* clone sensitive to suramin (TREU 1840) and its induced resistant derivate had similar karyotype patterns. Both clones had 11 chromosomal bands in the size ranges between 80 to 291 kb and 4 bands between 300 to 500 kb. The clone that was sensitive to Cymelarsan (TREU 1981), and its induced resistant derivate, had identical karyotype patterns with 9 chromosomal bands between 70 to 500 kb size range.

Neither similarities in the banding patterns, nor common bands shared between the cultured clones and the field isolates, were detected in this study.

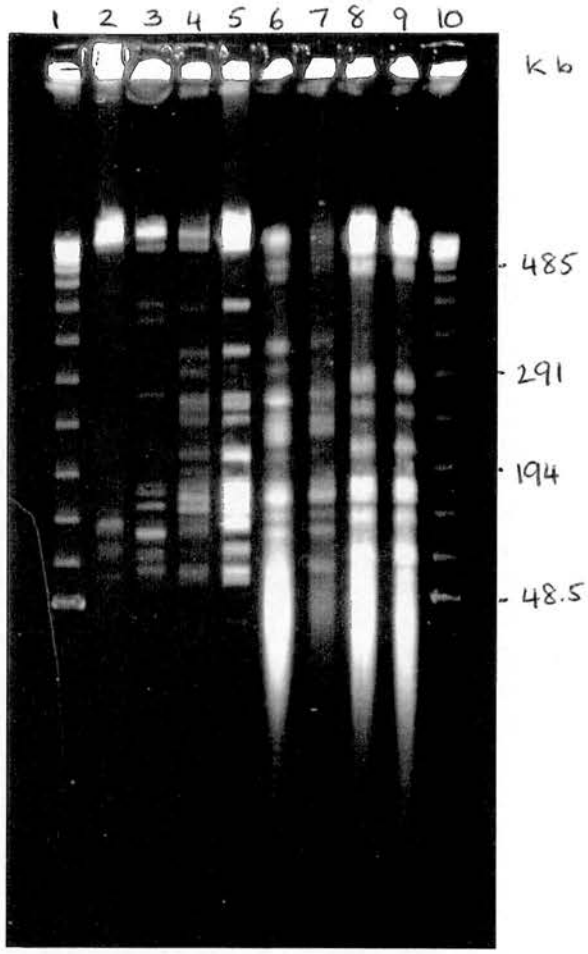


Figure 4.17. Karyotype patterns shown by *T. evansi* stocks which are resistant and sensitive to trypanocidal drugs separated by Transverse alternating field electrophoresis using running conditions to resolve DNA in the 50-500 bp size range.

Tracks 1, 10: Lambda DNA standard marker;
Track 2: BAKIT 294 (Suramin resistant)
Track 3: BAKIT 126 (Suramin resistant)
Track 4: BAKIT 134 (Suramin resistant)
Track 5: BAKIT 381 (Suramin resistant)
Track 6: SR1 (induced resistant to suramin)
Track 7: TREU 1840 (sensitive to suramin)
Track 8: MCR (induced resistant to cymelarsan)
Track 9: TREU 1981 (sensitive to cymelarsan)

4.4.4. DISCUSSION

This study did not detect any differences in the karyotype between the suramin resistant and sensitive clones and between Cymelarsan resistant and sensitive clones indicating that development of drug resistance in *T. evansi* has no detectable effect on the chromosome profile in the size range of 50-500 kb. The chromosome sizes observed in this study were intermediate and small chromosomes, which are subject to length alteration due to chromosome rearrangements associated with antigenic variations (Van der Ploeg *et al.*, 1984c). This study has suggested that direct karyotype analysis in this size range was unsuitable for detection of trypanocidal drug resistant stock. Karyotype separation in the chromosomes larger than 500 kb was not carried out due to the limited availability of the materials.

A different approach to detect karyotype polymorphisms in drug resistance and sensitive clones could be done by a combination of karyotype analysis and southern blot hybridisation with the gene probe detecting drug resistance. It was suggested that the presence of Malic enzyme type VII is associated with drug resistance in trypanosomes (Boid *et al.*, 1989). However, the gene associated with the expression of the enzyme has not been detected.

The presence of karyotype variability due to drug resistance in field conditions in Indonesia has not been detected. The four field stocks resistant to suramin were karyotyped using the electrophoresis conditions to separate bands between 50-900 kb size range, and showed different karyotype patterns. This result indicated that there was no single pattern for suramin resistance stocks.

4.5. EXPANDED SEPARATION OF *TRYPANOSOMA EVANSI* CHROMOSOMAL DNA BY TAFE

4.5.1. INTRODUCTION

The PFGE technique has been reported to have the capability of resolving almost the whole size range of chromosomal DNA. A *Leishmania major* clone was separated into approximately 23 bands in the size range of 250-2,000 kb (Samaras and Spithill, 1987). In contrast, *Leptomonas collosoma* was separated into 31 bands in the size range of 250-1650 kb (Beja *et al.*, 1994). *Trypanosoma cruzi* has been shown to contain at least 18 distinct bands in the 550 –1500 kb size range but no minichromosomes were detected (Aymerich and Goldenberg, 1989). Members of both the *Trypanozoon* and *Nannomonas (T.*

congolense) have the same general chromosomal organisation (Gibson & Borst, 1986), namely, intermediate and mini-chromosomes and very large DNA. *Trypanosoma brucei* was found to have approximately 100 mini-chromosomes in the size range 50-150 kb and at least 18 individual chromosomes with sizes up to 5.7 Mb (Van der Ploeg *et al.*, 1984a). The chromosomal DNA pattern of *T. evansi* stocks from Kenya has been determined and characterised by several discrete intermediate-sized chromosomes (150-600 kb) and large numbers of mini-chromosomes (50-150 kb) (Waitumbi & Young, 1994).

The present study was carried out to investigate the chromosome organisations in *T. evansi* stocks collected in Indonesia. Nine stocks representing 9 different karyotype patterns were chosen and subjected to the expanded TAFE conditions optimised to resolve chromosomal bands in the size range of: i) 0 - 100 kb; ii) 50 - 300 kb; iii) 50 - 500 kb; iv) 50 - 900 kb and v) 3.5 - 5.7 Mb.

4.5.2. MATERIALS AND METHODS

4.5.2.1. Trypanosomes

Nine *T. evansi* stocks isolated from buffalo and cattle in different areas in Indonesia (**Table 4.8**) were used for analysing the chromosome profiles using TAFE. The *T. evansi* DNA samples were embedded in agarose and prepared according to the method described in Chapter 3, Section 3.2.

Table 4.8. *Trypanosoma evansi* stocks used for the study on expansion of chromosomal DNA analysis by TAFE.

BAKIT No.	Karyotype Pattern	Karyotype Group
426	8	1.2
435	7	1.2
401	23	1.4
417	17	1.3
519	10	1.2
423	28	1.5
382	18	1.4
424	40	2
374	6	1.2

4.5.2.2. Transverse Alternating Field Electrophoresis

Agarose blocks of *T. evansi* DNA were separated by TAFE under conditions reported to resolve the chromosomes in the i) 0-100 kb; ii) 50-300 kb; iii) 50-500 kb; iv) 50-900kb and v) 3.5-5.7 Mb size ranges. Associated pulse times, voltage, currents and electrophoresis times required for these separations are detailed in **Table 4.9**.

All gels contained at least two tracks of the lambda DNA concatemer (BioRad, UK) as the size marker. All electrophoresis runs except the run to separate DNA in the 3.5-5.7 Mb size range were carried out under conditions of constant current. The separation run for DNA in the size range of 3.5-5.7 Mb was carried out at a constant voltage of 80 Volts. *Schizosaccharomyces pombe* (BioRad, UK) was used as the size marker.

Table 4.9. Optimised running conditions for TAFE II system.

Size range (Kb)	Running conditions					
	Stage	Duration (hours)	Pulse time		Current (mA)	Starting Voltage (Volts)
			minutes	seconds		
0-100 (1 stage)	1	18		8	275	320
50-300 (2 stages)	1	36		15	275	320
	2	8	30		10	
50-500 (3 stages)	1	8		15	275	320
	2	12		30	275	
	3	4		15	275	
50-900 (4 stages)	1	12		30	275	320
	2	12	1	30	275	
	3	12	2	30	275	
	4	8	30		10	10
3000-5700 (4 stages)	1	12	60			80
	2	24	45			
	3	24	30			
	4	8	30			10

4.5.2.3. Visualising the PFGE Gels

The gel staining, visualisation and photography procedures were described in section 4.2.2.4.3 and 4.2.2.4.4.

4.5.2.4. Separation of the Lambda Ladder by TAFE

Chromosomal band sizes were estimated by comparison to the fragment sizes separated in the lambda DNA standard marker. Graphs were drawn to show the relationships between the bands sizes and the distance travelled by the bands down the gel. The accuracy of the band sizes was examined by plotting the distance travelled down the gel by the lambda DNA fragments as a function of the molecular size of the marker. The function of the distance travelled by the lambda DNA fragments in each TAFE running condition was calculated by reciprocal fit.

4.5.3. RESULTS

4.5.3.1. Separation of the Lambda Ladder by TAFE

The band mobility of the lambda DNA fragments, separated by the optimised TAFE conditions, to resolve bands in the size range of 50-300 kb was illustrated in a graph shown in **Appendix 2.** The relationships between the bands sizes and the distance was shown by a straight line in the reciprocal fit, $y = 0.0008x + 0.0869$, $R^2 = 0.9773$.

The mobility plots of the lambda DNA fragments, separated by the TAFE optimised condition to resolve bands in the size range of 50-500 kb is represented by the reciprocal fit, $y = 0.0008x + 0.1276$, $R^2 = 0.97$ (**Appendix 3**).

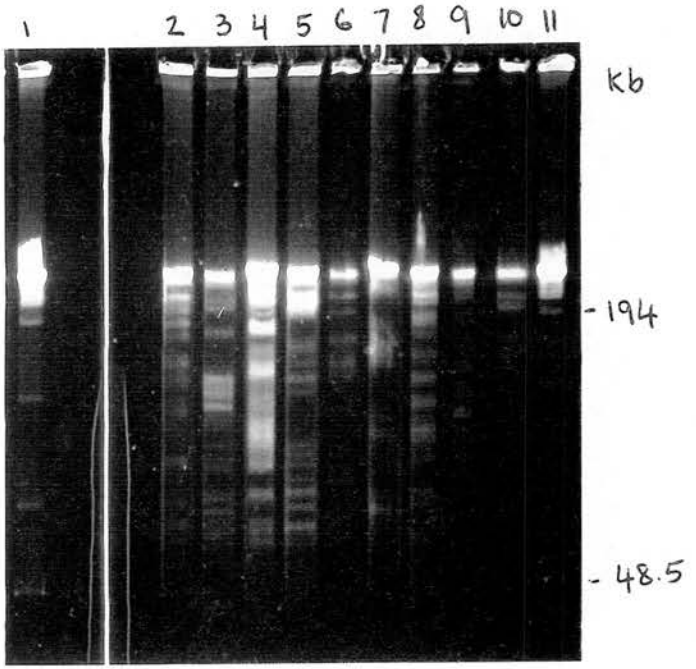
The mobility plots of the lambda DNA size marker separated by the TAFE conditions optimised to resolve bands between 50-900 kb size range are represented by the reciprocal fit, $y = 0.0002x + 0.1292$, $R^2 = 0.9909$, shown in **Appendix 4**.

4.5.3.2. Trypanosoma evansi *Karyotypes in 0-100 kb*

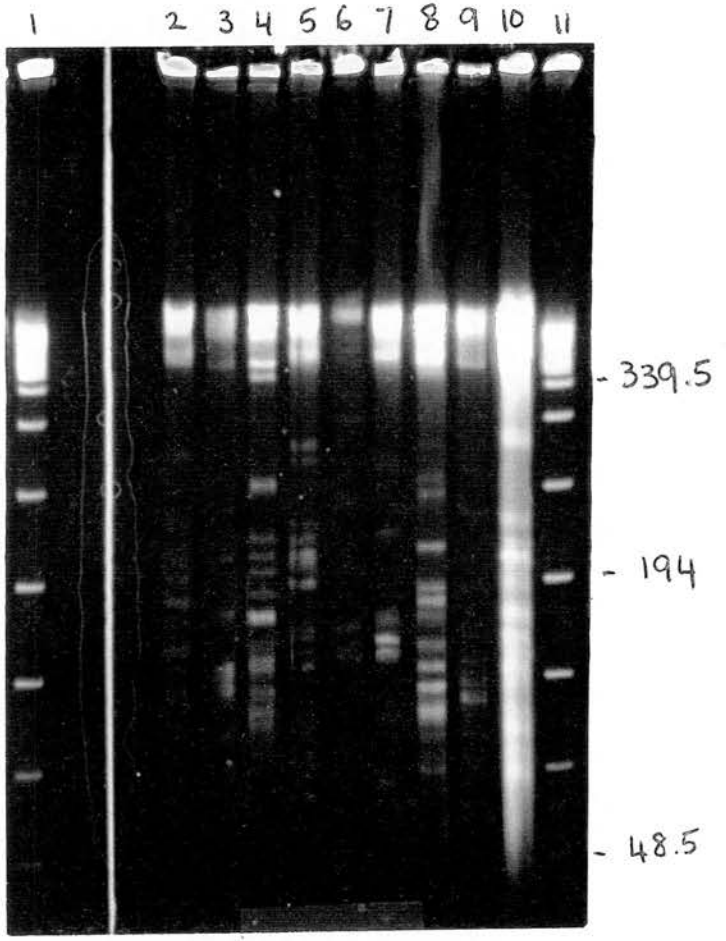
The TAFE running conditions specific for the DNA size range of 0-100 kb separated 4 DNA fragments of the lambda ladder size marker between 48.5 to 194 kb size range. Variations in the karyotype patterns were observed in the *T. evansi* stocks, none of them were identical (**Figure 4.18 A**). Chromosomal bands smaller than 50 kb were not detected in the stocks studied. Seven to 11 chromosomal bands were detected in the size range of 70-194 kb. Although the chromosomal banding patterns were not clearly resolved, the distribution of the

Figure 4.18 A and B. Chromosomal banding patterns from *Trypanosoma evansi* stocks separated by Transverse alternating field electrophoresis using running conditions optimised to resolve in the 0-100 kb (A); 50-300 kb (B) size range.

Tracks 1, 11: Lambda DNA concatemer size standard
Track 2: BAKIT 426 (group1.2)
Track 3: BAKIT 435 (group 1.2)
Track 4: BAKIT 401 (group 1.4)
Track 5: BAKIT 417 (group 1.3)
Track 6: BAKIT 519 (group 1.2)
Track 7: BAKIT 423 (group 1.5)
Track 8: BAKIT 382 (group 1.4)
Track 9: BAKIT 424 (group 2)
Track 10: BAKIT 374 (group 1.2)



A



B

chromosomal bands can be divided into 3 size classes: a) chromosomes that remained in the gel slot; b) chromosomes in the compression zone; c) small chromosomes in the size range of 50-200 kb.

4.5.3.3. Trypanosoma evansi *Karyotypes in 50-300 kb*

The TAFE conditions separated 7 DNA fragments in the lambda size marker (48.5-339.5 kb). The karyotype patterns separated by TAFE were variable and none of them were identical (**Figure 4.18 B**). In general, the chromosome organisation of *T. evansi* were separated into 4 major size classes: a) chromosomes that stayed in the gel slot; b) chromosomal bands which remained in the compression zone; c) 3 to 10 intermediate sized chromosomes of larger than 145 kb to 250 kb and d) 2 to 6 minichromosomal bands in the size range of 80-145 kb. Chromosomal bands were not found in the sizes smaller than 80 kb in the stocks studied. Good banding pattern resolution was observed in chromosomal bands in the 150-300 kb size range.

4.5.3.4. Trypanosoma evansi *Karyotypes in 50-500 kb*

The optimised TAFE conditions to separate bands in the size range of 50-500 kb resolved 10 lambda DNA fragments (48.5-485 kb). These optimised TAFE conditions separated 8 to 11 *T. evansi* chromosome bands within the 80 to 500 kb size range with varied patterns in different stocks (**Figure 4.18 C**). In general, the *T. evansi* chromosome profiles were divided into 4 major size classes: a) chromosomes remained in the gel slot; b) chromosomes remained in the compression zone; c) 2 to 7 intermediate sized chromosomes between 200-500 kb and d) at least 4 minichromosomal bands smaller than 200 kb. Poor band resolutions of minichromosomes of smaller than 150 kb were observed in the stocks studied.

4.5.3.5. Trypanosoma evansi *Karyotypes in 50-900 kb*

Seventeen lambda DNA fragments (48.5 - 824.5 kb) were separated by the optimised TAFE conditions to resolve bands between 50-900 kb size range. The TAFE separated *Trypanosoma evansi* chromosomes to 8-13 bands in the 80-900 kb size range (**Figure 4.18 D**). The organisations of *T. evansi* chromosomes were divided into 4 major size classes: a) chromosomal DNA remained in the gel slot; b) Megabase-sized chromosomes which remained in the compression zone; c) 3-8 intermediate size chromosome bands in the size range of 200-900 kb and d) at least 3 or 4 or a smear of mini-chromosomal bands of smaller than 150 kb. Neither identical karyotype patterns nor common bands shared by all of the 9 *T. evansi* stocks were observed.

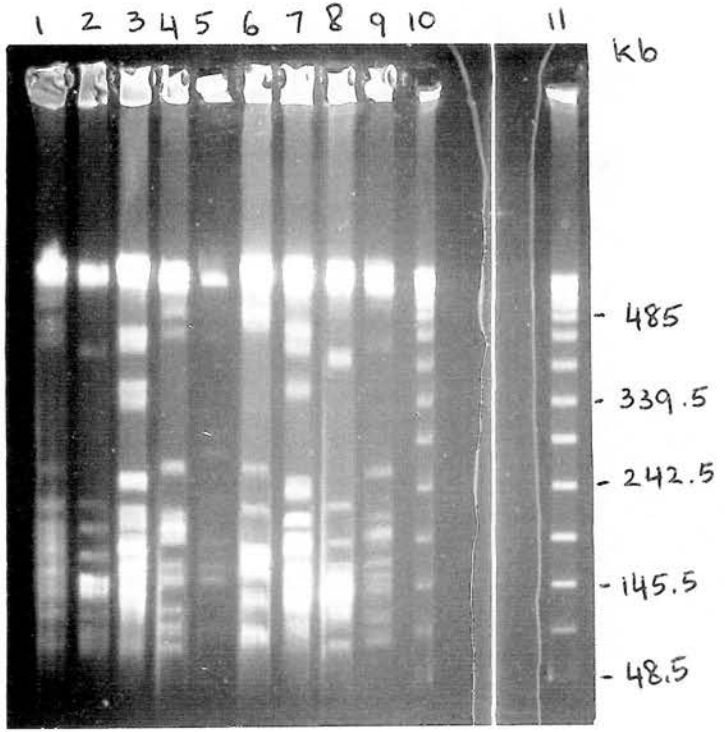
Figure 4.18 C and D. Chromosomal banding patterns from *Trypanosoma evansi* stocks separated by Transverse alternating field electrophoresis using running conditions optimised to resolve in the 50-500 kb (C); 50-900 kb (D) size range.

Figure 4.18 C

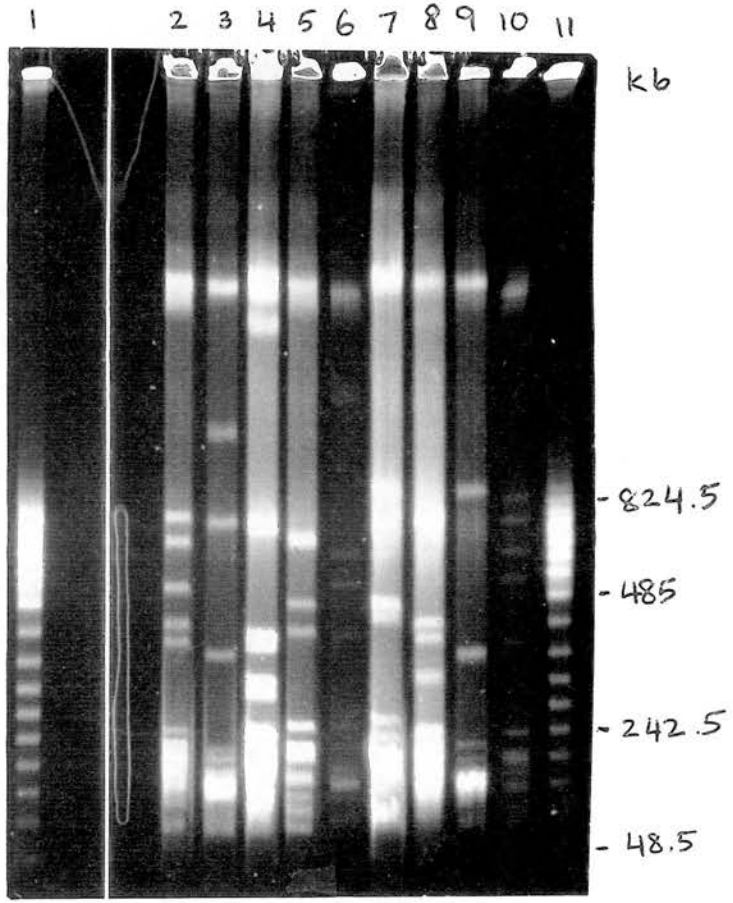
Tracks 10, 11: Lambda DNA concatemer size standard
Track 1: BAKIT 426 (group 1.2)
Track 2: BAKIT 435 (group 1.2)
Track 3: BAKIT 401 (group 1.4)
Track 4: BAKIT 417 (group 1.3)
Track 5: BAKIT 519 (group 1.2)
Track 6: BAKIT 423 (group 1.5)
Track 7: BAKIT 382 (group 1.4)
Track 8: BAKIT 424 (group 2)
Track 9: BAKIT 374 (group 1.2)

Figure 4.18 D

Tracks 1, 11: Lambda DNA concatemer size standard
Track 2: BAKIT 426 (group1.2)
Track 3: BAKIT 435 (group 1.2)
Track 4: BAKIT 401 (group 1.4)
Track 5: BAKIT 417 (group 1.3)
Track 6: BAKIT 519 (group 1.2)
Track 7: BAKIT 423 (group 1.5)
Track 8: BAKIT 382 (group 1.4)
Track 9: BAKIT 424 (group 2)
Track 10: BAKIT 374 (group 1.2)


1 2 3 4 5 6 7 8 9 10 11
kb
485
339.5
242.5
145.5
48.5

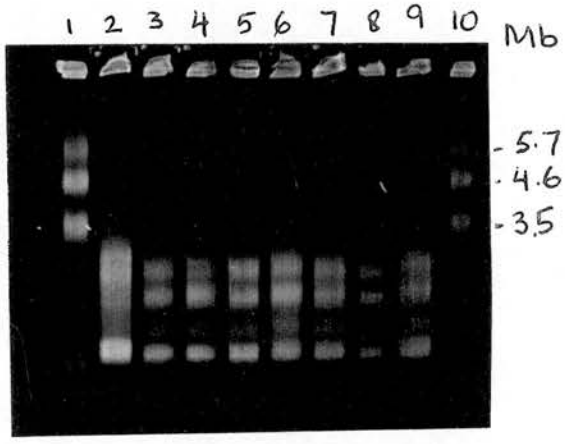
C


1 2 3 4 5 6 7 8 9 10 11
kb
824.5
485
242.5
48.5

D

Figure 4.18 E. Chromosomal banding patterns from *Trypanosoma evansi* stocks separated by Transverse alternating field electrophoresis using running conditions optimised to resolve in the 3.5-5.7 Mb size range.

Tracks 1, 10: *Schizosaccharomyces pombe* size standard
Track 2: BAKIT 505 (Group 1.1)
Track 3: BAKIT 513 (Group 1.2)
Track 4: BAKIT 509 (Group 1.2)
Track 5: BAKIT 508 (Group 1.2)
Track 6: BAKIT 502 (Group 1.2)
Track 7: BAKIT 499 (Group 1.2)
Track 8: BAKIT 498 (Group 1.2)
Track 9: TREU 2225.


1 2 3 4 5 6 7 8 9 10
Mb
5.7
4.6
3.5

4.5.3.6. Trypanosoma evansi Karyotypes in 1-5.7 Mb

The TAFE separated 3 chromosomal bands in *S. pombe* standard marker: 3.5, 4.6 and 5.7 Mb. However, the *T. evansi* Megabase-sized chromosomes were not well resolved by these conditions. Typical separation of the large chromosomes of 9 *T. evansi* stocks is shown in (**Figure 4.18 E**). Two to three bands located between 3.5 to 5.7 Mb and four to five bands of smaller than 3.5 Mb. The chromosome profiles shown in the size range of 1-5.7 Mb showed less variety in their banding patterns than those separated in smaller size range (50 kb -< 1 Mb).

4.5.4. DISCUSSION

The results of the present study have shown that variation in TAFE running conditions separates chromosomal bands of *T. evansi* in different size ranges. Seven to 11 minichromosomal bands (between 70-194 kb size range) were separated using the optimised running conditions to separate bands in the 0-100 kb size range. Using the optimised conditions to separate bands in the 50 – 500 kb size range, however, poorer minichromosomal bands resolution was obtained. The poor resolutions, or a smear of minichromosomes, are often found in optimised TAFE conditions to separate large molecular size, as shown in **Figure 4.18D**. The band smear separated by the 50 – 900 kb TAFE conditions appeared to consist of 7 to 11 bands between 80 – 200 kb size range. The presence of poor resolutions, or band smear, is caused by band stacking, as the DNA fragments move slower near the bottom of the gel. In the TAFE system the reorientation angle is 115° on the top of the gel resulting in a clear resolution of large DNA bands between 200 – 300 kb size range (Lai *et al.*, 1989). However, the reorientation angle is much greater (165°) at increasing distances from the wells (Birren and Lai, 1993, Gardiner, 1992). This causes slower mobility of DNA down the gel resulting in band stacking near the bottom of the gel.

The intermediate sized chromosomes were clearly resolved by the optimised TAFE conditions used to separate bands between 50 – 900 kb. Although these running conditions clearly resolved the DNA ladder between 48.5 to 242.5 kb, the separation of *T. evansi* bands between this size range was poorly resolved. This may be due to the high numbers of chromosomal bands present in 50 – 200 kb size range in *T. evansi.* It was difficult, however, to estimate the exact numbers of chromosomes in *T. evansi* due to the non-stoichiometric ethidium bromide staining with the DNA bands.

The results from this study showed that the resolution of the banding patterns of chromosomes of the megabase size was not clear although they were separated under

appropriate TAFE conditions as recommended by Dih and Morgenstern (1990) for GeneLine II *S. pombe* separation. It was suggested that the separation of the fragments in the size ranges larger than 1600 kb are more sensitive to the PFGE conditions than are the smaller fragments than 1600 kb, therefore, it was also suggested to control the voltage, current, temperature and buffer composition to get the reproducible resolutions of the Megabase-size ranges (Gardiner, 1992). Small changes (10%) in the voltage condition, causes the two largest bands in *S. pombe* unresolved, however, small increase might cause band trapping and yield undetectable bands (Gardiner, 1992). It seemed that the weak resolutions of the *S. pombe* chromosomes in this study caused by the small increase of the voltage condition, despite of the PFGE conditions were set appropriately. Causes of the changes, if any, in the voltage condition in the TAFE system used in this study remained unknown.

The present study demonstrated that alternation of the pulse times in the TAFE running conditions had changed the separation of the band size ranges. Short pulse times separated small molecules, for example, an 8 second pulse time in the gel, run for 18 hours, separated the *T. evansi* mini-chromosomes in the size range of 0-150 kb (**Figure 4.18 A**). Longer electrophoresis times (24 or 36 hours) did not generate better separation and decrease the resolutions of smaller molecules because of the bands are stacking near the bottom of the gel, loosing separation (Gardiner, 1992).

Results from this study have shown the variation in the number of chromosomal bands separated by different TAFE conditions. A complete resolution of all chromosomes of *T. evansi* was difficult to achieve under the TAFE running conditions used in the study. The presence of DNA in the sample well was observed in all gels, which was unresolved using different TAFE running conditions.

The separation of large chromosomes (>2 Mb) may need longer electrophoresis time. Henriksson *et al.* (1995) found that a complete separation of all chromosomes of *T. cruzi* was not possible even if the PFGE conditions were optimised for separating different chromosomal band size ranges.

4.6. STUDIES ON CHROMOSOME POLYMORPHISM AND VARIANT ANTIGENIC TYPES IN RELAPSE POPULATIONS OF *TRYPANOSOMA EVANSI*

4.6.1. INTRODUCTION

Variations in both chromosomal size, and chromosomal distribution, of particular genes between trypanosomes, were first demonstrated by Van der Ploeg *et al.* (1984b, c), Gibson

et al. (1985) and Gibson and Miles (1986). Results from the present study on chromosome profiles analysis of *T. evansi* stocks from Indonesia showed a high degree of variability in the small and intermediate size chromosomes (50-900 kb size range). Karyotype analysis proved to be a useful tool for stock differentiation of *T. evansi* isolates in Indonesia. However, trypanosome chromosomes change their sizes even in a single relapse infection (Van der Ploeg *et al.*, 1984c). Therefore the stability of the karyotype patterns can be called into question.

Changes in the intermediate size chromosomes (200-700 kb) of *T. brucei* appeared to be associated with changes in the surface antigen genes expression (Van der Ploeg *et al.*, 1984c), as many of the variant surface glycoprotein genes are located on chromosomes sized 50-700 kb (Van der Ploeg *et al.*, 1984a). Evidence of the variant surface glycoprotein gene expression site switches, which resulted in size alteration of chromosomes, was reported by Shea *et al.* (1986) in 3 *T. brucei* variants derived serially from single relapse infections.

The high degree of karyotype polymorphism in *T. evansi* in Indonesia, described in section 4.3.3.1, would be due to the frequent chromosome translocations resulting from changes in the expression sites of surface antigen genes as had been reported by Van der Ploeg *et al.* (1984c) in *T. brucei.* However, the karyotype analysis carried out in the study yielded better chromosomal banding patterns resolution than those performed on *T. brucei* (Van der Ploeg *et al.*, 1984a,c), or *T. evansi* in Kenya (Waitumbi and Young, 1994), which could explain the high degree of karyotype variation in the Indonesian *T. evansi* stocks. This study was carried out to establish one of the possible causes of the wide karyotype differences in *T. evansi* stocks. Two separate studies were carried out to determine the relationships between antigenic variation and the chromosome polymorphism in relapse populations derived from infections with a *T. evansi* field stock in one experiment and with a clone of the same stock of *T. evansi* in the second experiment.

4.6.2. MATERIALS AND METHODS

4.6.2.1. Trypanosomes

Two stocks were used, a *T. evansi* field stock originating from Central Java (BAKIT 374) was used in the first study. The second study used a cloned population of the same *T. evansi* stock, TREU 2253.

4.6.2.2. Infections

4.6.2.2.1. Experiment 1

The first part of the study used materials already available at CTVM (Boid, *et al.*, 1992) from previous studies on *T. evansi*. Briefly, a *T. evansi* stock (TREU 2225) was used to infect a rabbit and trypanosome populations and serum samples were collected 7, 11, 14, 18 and 21 days post infection. Blood and serum samples were taken before parasite inoculation and additionally at 28 days post infection. Each relapse population was cloned in mice (Cross, 1975) and cryopreserved as a stabilate population.

4.6.2.2.2. Experiment 2

A clone (TREU 2253) was prepared from a cryopreserved population expanded in mice of BAKIT 374/Central Java and used to infect two rabbits. Each rabbit was inoculated intravenously with infected mouse blood containing 10^6 trypanosomes. Blood and serum samples were collected daily from day 4 to 14 post infection. Blood samples were examined for the presence of *T. evansi* infection by the microhaematocrit centrifugation technique (MHCT) developed by Woo (1970) and subinoculated to mice. After passage in the mice stabilates were prepared from high parasitaemic mouse blood 3 or 4 days after inoculation.

4.6.2.3. Production of Cloned Population

Clones of relapse populations were prepared according to Cross (1975) with minor procedure modifications (Jones, pers. comm.). Infected mouse blood was serially diluted (10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) in phosphate buffered saline (95 mM Na_2HPO_4 anhydrous, 5 Mm NaH_2PO_4, 72.7 mM NaCl) containing 10% foetal calf serum and kept on ice. Six droplets (approximately 0.5 µl) of the highest dilution were deposited onto a microscope slide (22 x 64 mm) then the slide was turned upside down and placed on a square plastic chamber (22 x 55 mm) containing water to maintain high moisture levels. The droplet was quickly examined by a dark-field illumination microscope at a magnification (x 100) that enabled the droplet to be viewed in a single microscope field. A droplet seen to contain a single parasite was drawn up using a 1 ml disposable syringe already filled with approximately 100 µl PSG and immediately injected into a mouse intraperitoneally. Five mice were infected for each clone. Pre-treated (24 hours before inoculation) cyclophosphamide (3 grams per kg body weight) mice (Smith, Levine and Mansfield, 1982) were used when the parasite grew slowly in primary infected mice. Mice were examined for the presence of trypanosomes at 3 or 4 days after inoculation by examination of the mouse tail blood using MHCT of Woo (1970) followed by subinoculations of the whole blood from trypanosome positive mice. On some

occasions, however, a 'blind passage' was performed when parasitaemia was not observed in the mouse by 4 days post infection. After an appropriate number of passages stabilates were prepared from high parasitaemic mouse blood. Procedures for stabilate preparations have been described in Chapter 3, Section 3.1.4.

4.6.2.4. Transverse Alternating Field Electrophoresis

Agarose block sample inserts were prepared from relapse populations and their corresponding clones collected from both experiments. Procedures of agarose blocks preparations have been described in Chapter 3, Section 3.2.

Transverse alternating field electrophoresis was performed with these populations, using the two optimised PFGE conditions to resolve chromosomal bands in the size range of 50-500 kb and 50-900 kb. Visualisation of the ethidium bromide gel after PFGE and determination of the band sizes have been described in previous section (Section 4.2.2.4).

4.6.2.5. Agglutination Test for Identification of Antigenic Variants of T. evansi

The agglutination test for trypanosomes (AGT) was performed according to Lumsden *et al.*, (1973) to determine the antigenic relationships of the relapse populations and their corresponding clones.

To establish antigenic relationships each population was tested against all the serum samples collected from the rabbit. The primary isolate (BAKIT 374) was also included in the test. One capillary of appropriate stabilate population was diluted in 200 μl or 150 μl borate buffer (0.11 M Borax and 0.31 M Boric acid), to achieve the concentration of approximately 10^7 trypanosomes/ml or as indicated by the presence of 15-20 trypanosomes per microscope field examined by 400 x magnification.

Serum samples were serially diluted from 1:2 to 1:64 in borate buffer. Normal serum and the antigens were tested against the borate buffer as controls.

For the agglutination assay, a microplate (NUNC HLA Plate) was filled with 5 ml mineral oil (Light weight oil, Sigma Chemical Co. St. Louis, USA) so that the wells were covered with the oil. Each well was then loaded with 2 μl of an appropriate serum dilution; the first row of each plate being reserved for control wells (buffer + antigen). The trypanosome suspension was pipetted as 2 μl into each well, ensuring that it mixed with the serum, then the plate was incubated for 1 hour at 37^0 C. Examination of the wells was carried out using dark field microscopy (100 x) for the presence of agglutinating trypanosomes seen as a light cluster against a dark background.

4.6.3. RESULTS

4.6.3.1. Experiment 1

One clone was prepared from each of the corresponding 5 relapse populations isolated during 21 days infection in rabbit (**Table 4.10**). Each population had a different antigenic variant, which was shown by the agglutination test.

Table 4.10. *Trypanosoma evansi* stabilates produced from relapse populations and their clones isolated from an experimental infection in rabbit.

TREU No.	Days post infection	Relapse/Clone
2225	0	Uncloned
2253		Clone of TREU 2225
2232	7	Relapse 1 of infection with TREU 2225
2292		Clone of 2232
2256	11	Relapse 2 of infection with TREU 2225
2293		Clone of TREU 2256
2261	14	Relapse 3 of infection with TREU 2225
2295		Clone ofTREU 2261
2257	18	Relapse 4 of infection with TREU 2225
2297		Clone of TREU 2257
2258	21	Relapse 5 of infection with TREU 2225
2294		Clone of TREU 2258

4.6.3.1.1. Agglutination test for identification of antigenic variants of *T. evansi*

The cloned and uncloned populations generated from relapse infections showed no differences in their reaction pattern in the trypanosome agglutination test against serum samples collected at 7, 11, 14, 18, 21 and 28 days post infection (**Table 4.11**). The original stock (BAKIT 374), designated as population of day 0 and its clone, population day 0_C, reacted with all serum collected at relapse infections. The relapse trypanosome populations collected at 7 days post infection and its clone (7_C) did not react with the serum collected at the same time but reacted to serum collected later. Similar reactions were also seen in other populations collected at 11, 14 and 18 days post infection and their corresponding clones that agglutinated by the serum collected after the corresponding population was isolated. Trypanosome populations collected at 21 days post infection and its clone (21_C), however, reacted to the serum collected at the same time of its isolation. These results indicate that

each relapse population was antigenically different, as it was only agglutinated by the serum collected after its isolation and each population showed a different agglutination pattern with the 6 sera collected.

Table 4.11. Experiment 1: Summary of the results on Trypanosome Agglutination Test (AGT) on *T. evansi* stocks isolated from relapse populations in rabbit infected with *T. evansi* TREU 2253.

Day Infection	Antigen (TREU)	Trypanosome Agglutination Test Results Serum samples (day post infection)						
		N	7	11	14	18	21	28
0	2225	-	+	+	+	+	+	+
0_C	2253	-	+	+	+	+	+	+
7	2232	-	-	+	+	+	+	+
7_C	2292	-	-	+	+	+	+	+
11	2256	-	-	-	+	+	+	+
11_C	2293	-	-	-	+	+	+	+
14	2261	-	-	-	-	+	+	+
14_C	2295	-	-	-	-	+	+	+
18	2257	-	-	-	-	-	+	+
18_C	2297	-	-	-	-	-	+	+
21	2258	-	-	-	-	-	+	+
21_C	2294	-	-	-	-	-	+	+

N = Normal serum (day 0).
$_C$ = Clone of the corresponding relapse population
+ = Agglutination present.
- = No agglutination observed.

4.6.3.1.2. Transverse alternating field electrophoresis

Karyotype analysis showed that the karyotype patterns of the trypanosome populations appear to cycle. The karyotype pattern I, which was the pattern of the original stock used for inoculation, changed to pattern II at 7 days after infection, and changed to pattern III after 11 days but changed back to pattern I after 14 and 21 days post infection. It was noted that karyotype III (the 11 day population) was a mixture of karyotypes I and II.

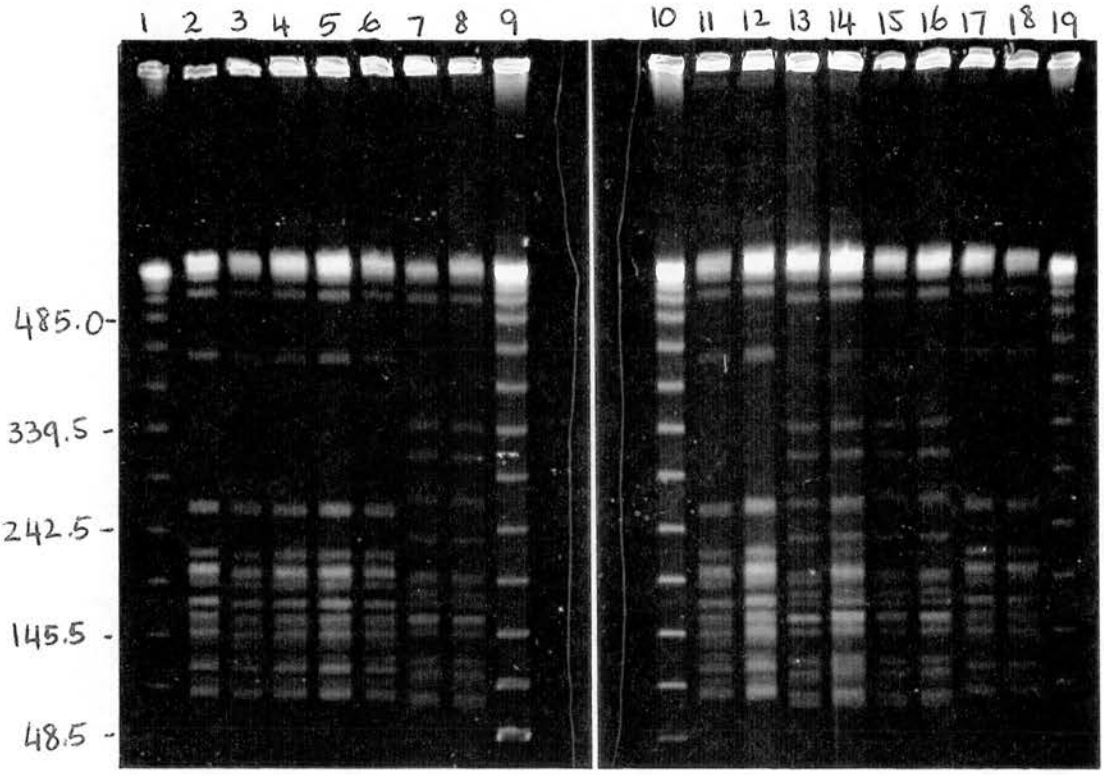

1 2 3 4 5 6 7 8 9
10 11 12 13 14 15 16 17 18 19
485.0
339.5
242.5
145.5
48.5

Figure 4.19. Chromosomal banding patterns of the relapse populations of *T. evansi* separated by Transverse alternating field electrophoresis using the running conditions to resolve bands in the size range of 50-500 kb.

Tracks 1, 9, 10, 19: Lambda DNA concatemer size standard
Track 2: BAKIT 374 (primary isolate, day 0; Pattern I)
Track 3: TREU 2225 (day 0; Pattern I)
Track 4: TREU 2253 (Clone; day 0; Pattern I)
Track 5: TREU 2294 (Clone; day 21; Pattern I)
Track 6: TREU 2258 (day 21; Pattern I)
Track 7: TREU 2297 (Clone; day 18;Pattern II)
Track 8: TREU 2257 (day 18; Pattern II)
Track11: TREU 2295 (Clone; day 14; Pattern I)
Track12: TREU 2261 (day 14; Pattern I)
Track13: TREU 2293 (Clone; day 11; Pattern II)
Track14: TREU 2256 (day 11; Pattern III)
Track15: TREU 2292 (Clone; day 7; Pattern II)
Track16: TREU 2232 (day 7; Pattern II)
Track17: TREU 2253 (Clone; day 0; Pattern I)
Track18: TREU 2225 (day 0; Pattern I)

Karyotypes of the relapse populations between 50-500 kb

The Transverse alternating field electrophoresis separated 12-15 *T. evansi* chromosomal bands in the relapse populations between 80-450 kb size range (**Figure 4.19**). Three karyotype patterns were detected in the 5 relapse populations, however their corresponding clones only had 2 patterns (**Table 4.12**). The chromosome profiles of the uncloned relapse population were identical to their corresponding clones, apart from the population collected at 11 days post infection that showed a different pattern from its clone. The chromosomal banding pattern characteristic of patterns I, II and III of the relapse population are presented in **Table 4.13**.

Table 4.12. Karyotype pattern variability of *Trypanosoma evansi* relapse populations and their corresponding clones separated by pulsed-field gel electrophoresis.

Relapse population (day post infection)	Karyotype Pattern	
	Relapse	Clone
0 (Original stock)	I	I
7 (Relapse 1)	II	II
11 (Relapse 2)	III	II
14 (Relapse 3)	I	I
18 (Relapse 4)	II	II
21 (Relapse 5)	I	I

Karyotypes of the relapse populations between 50-900 kb

The TAFE separated 11 to 13 chromosomal bands in the *T. evansi* relapse populations in the size range of 80-850 kb (**Figure 4.20**). Analysis on the karyotype patterns identified the same 3 groups as in the 50-500 kb bands size range. The chromosomal banding pattern characteristic of patterns I, II and III of the relapse populations are presented in **Table 4.13**.

Figure 4.20. Chromosomal banding patterns of the relapse populations of *T. evansi* separated by Transverse alternating field electrophoresis using the running conditions to resolve bands in the size range of 50-900 kb.

Tracks 1, 10, 11, 20: Lambda DNA concatemer size standard
Track 2: TREU 2225 (day 0; Pattern I)
Track 3: BAKIT 374 (primary isolate, day 0; Pattern I)
Track 4: TREU 2225
Track 5: TREU 2253 (Clone; day 0; Pattern I)
Track 6: TREU 2294 (Clone; day 21; Pattern I)
Track 7: TREU 2258 (day 21; Pattern I)
Track 8: TREU 2297 (Clone; day 18;Pattern II)
Track 9: TREU 2257 (day 18; Pattern II)
Track12: TREU 2295 (Clone; day 14; Pattern I)
Track13: TREU 2261 (day 14; Pattern I)
Track14: TREU 2293 (Clone; day 11; Pattern II)
Track15: TREU 2256 (day 11; Pattern III)
Track16: TREU 2292 (Clone; day 7; Pattern II)
Track17: TREU 2232 (day 7; Pattern II)
Track18: TREU 2253 (Clone; day 0; Pattern I)
Track19: TREU 2225 (day 0; Pattern I)

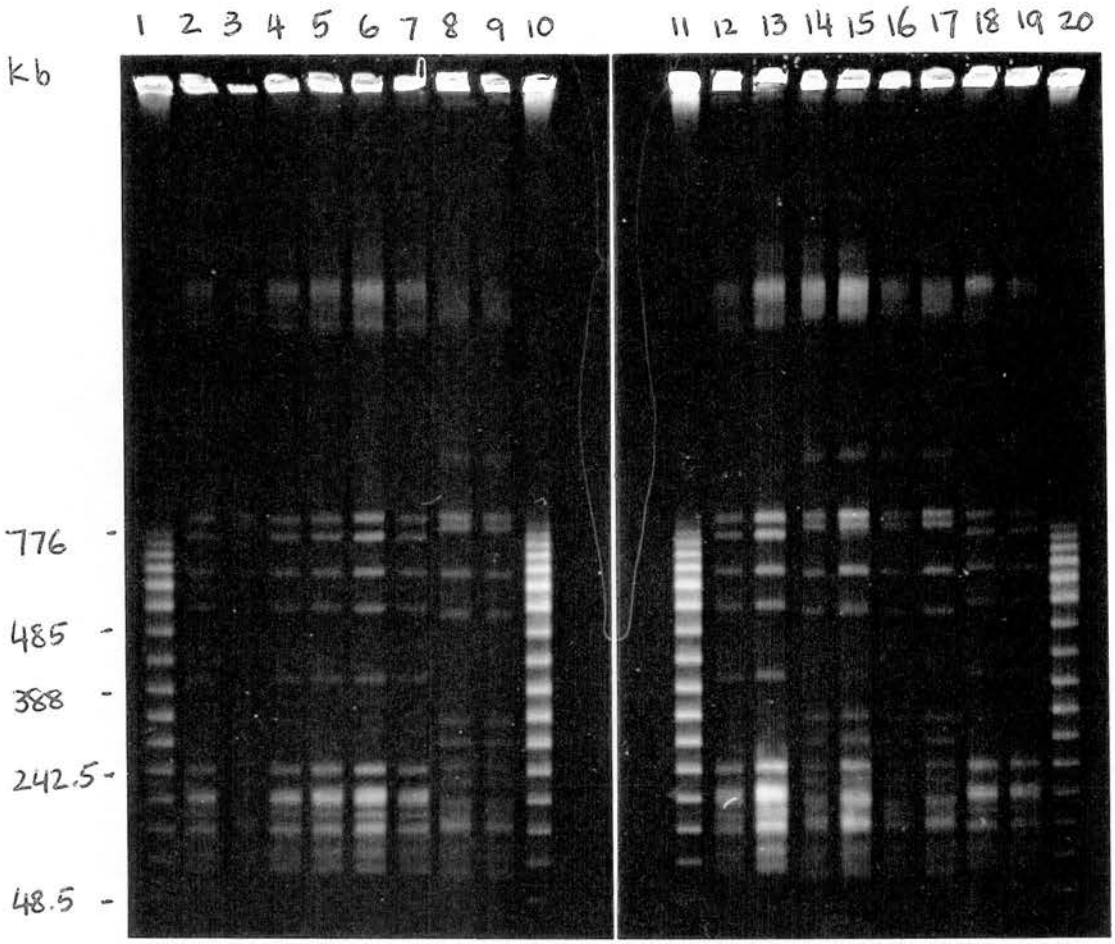

1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
kb
776
485
388
242.5
48.5

Table 4.13. Characteristic of chromosomal banding patterns (Pattern I, II and III) of relapse populations.

Karyotype Pattern	50-500 kb			50-900 kb		
	Total no of bands	Band size (kb)	No of bands	Total no of bands	Band size (kb)	No of bands
I	13	90-220	11	11	80-240	6
		260	1		400-850	5
		420	1			
II	14	80-200	10	12	80-330	8
		230-340	4		520-850	4
III	16	80-200	10	13	80-330	8
		230-340	5		400	1
		420	1		520-850	4

4.6.3.2. Experiment 2

Experiment-2 was carried out to examine the karyotype polymorphism in relapse populations isolated from an infection with a cloned stock produced from the same stock used in experiment-1 using similar protocols, except that only one TAFE condition was used (50-900 kb size range separation).

4.6.3.2.1. Agglutination test for identification of antigenic variants of *T. evansi*

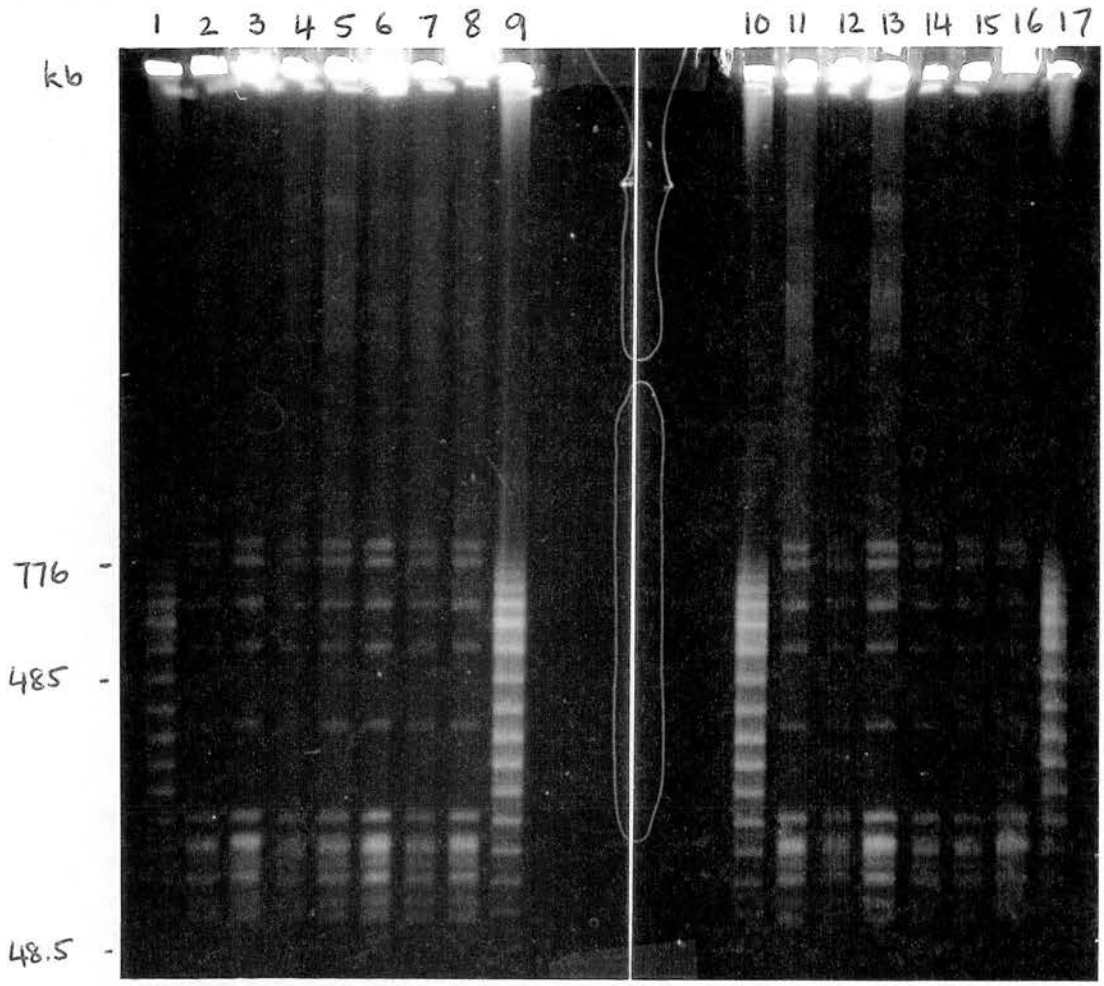
Six different agglutination patterns were detected in the relapse population collected during 14-day period of infection (**Table 4.14**). The clone used for infection (day 0_C population) was agglutinated by all of the serum samples collected. Each relapse population isolated at 4-11 days after infection agglutinated with the serum collected 2-5 days after its collection. The relapse populations isolated between 6-11 days post infection showed similar agglutination patterns. The population isolated at 12 and 13 days post infection was agglutinated by all of the serum, however, the 14-day population did not show clear agglutination reactions with any of the serum.

Table 4.14. Experiment 2: Summary of the results of Trypanosome Agglutination Test (AGT) on *T. evansi* stocks isolated from relapse populations in a rabbit experimentally infected with TREU 2317 (=TREU 2253) compared with the chromosomal banding pattern showed by the pulsed-field gel electrophoresis (PFGE). N= Normal serum; -= No agglutination; ± = < 50% agglutination; += ≈ 60-70% agglutination; ++= ≈ 80-90% agglutination; +++= 100% agglutination; (a)= 1:2 serum dilution; (b)= 1:4 serum dilution; (c)= 1:8 serum dilution; (d)= 1:16 serum dilution.

PFGE Pattern	Trypanosome Agglutination Test (AGT) Results															
	AGT Pattern	Day Infection	Antigen (TREU)	Day infection serum samples												
				N	4	5	6	7	8	9	10	11	12	13	14	18	21
I	I	0	2317	± (b)	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++
I	II	4	2320	-	-	-	-	-	±	±	+	+++	+++	+++	+++	+++	+++
I	III	5	2324	± (a)	± (a)	± (a)	± (c)	±	+++	+++	+++	+++	+++	+++	+++	+++	+++
I	IV	6	2327	± (a)	± (a)	± (a)	± (a)	± (a)	± (a)	± (a)	± (c)	+++	+++	+++	+++	+++	+++
I	IV	7	2323	-	-	-	± (a)	± (a)	± (a)	± (a)	± (a)	+	+	++	+++	+++	+++
I	IV	8	2326	-	-	-	-	-	-	± (a)	± (a)	+	+++	+++	+++	+++	+++
I	IV	9	2329	-	± (b)	± (b)	± (b)	± (b)	± (b)	± (d)	± (d)	+++	+++	+++	+++	+++	+++
I	IV	10	2321	-	± (a)	± (a)	± (a)	± (a)	± (a)	± (a)	± (a)	±	+	++	++	+++	+++
I	IV	11	2336	± (b)	± (a)	± (a)	± (a)	± (a)	± (a)	± (a)	± (a)	±	±	±	++	++	+
I	V	12	2334	+	+	+	+	+	+	+	+	+	+	+	++	+	+
I	V	13	2338	±	±	±	±	±	±	±	±	±	±	±	+	+	+
I	VI	14	2340	-	-	-	-	-	-	-	-	-	-	± (a)	± (c)	± (c)	± (d)

Figure 4.21. Karyotype patterns of *T. evansi* relapse populations separated by Transverse alternating field electrophoresis using the running conditions to resolve DNA in the 50-900 kb size range. The *T. evansi* populations were collected daily from day 4 to 14 after infection with a *T. evansi* clone (TREU 2253).

Track 1, 9, 10, 17: Lambda DNA concatemer size standard
Track 2: TREU 2253 (Cloned population used for infection)
Track 3: TREU 2320 (Day 4)
Track 4: TREU 2324 (Day 5)
Track 5: TREU 2327 (Day 6)
Track 6: TREU 2323 (Day 7)
Track 7: TREU 2326 (Day 8)
Track 8: TREU 2329 (Day 9)
Track11: TREU 2321 (Day 10)
Track12: TREU 2336 (Day 11)
Track13: TREU 2334 (Day 12)
Track14: TREU 2338 (Day 13)
Track15: TREU 2340 (Day 14)
Track16: TREU 2225 (uncloned, primary isolate/ Day 0)


1 2 3 4 5 6 7 8 9
10 11 12 13 14 15 16 17
kb
776
485
48.5

4.6.3.2.2. Transverse alternating field electrophoresis

The pulsed-field gel electrophoresis separated 11 chromosomal bands in the size range of 80-850 kb (**Figure 4.21**). Only one banding pattern was seen in all of the populations that were identical to karyotype pattern of the infecting population.

4.6.4. DISCUSSION

Infection with an uncloned stock of *T. evansi* produced a series of populations with different karyotype patterns that appeared to be related to the appearance of antigenic variants. The karyotype polymorphism in the relapse population examined in this study might represent the chromosome rearrangements due to the varieties in the antigenic variation, as had been shown in a single relapse experiment described by Van der Ploeg *et al.* (1984c). Drastic changes in the intermediate chromosomes by alternate appearance/disappearance of a band detected in 3 variants derived serially from one another in single relapses (Van der Ploeg *et al.*, 1984c; Shea *et al.*, 1986). It is more likely, however, that the karyotype polymorphism in *T. evansi* relapse populations due to the mixed karyotypes contained in the uncloned original stock used for infection, as infection with a cloned stock resulted in population with similar banding patterns.

Results from this study have shown that karyotype pattern is stable within populations of the same genetic background and are unaffected by the process of antigenic variation. The appearance of different banding patterns in sequential population from uncloned stocks was considered to be due to the presence of an organism with at least two different genetic backgrounds.

4.7. CHROMOSOME PROBE PRODUCTION

4.7.1. INTRODUCTION

The study on the characterisation of *T. evansi* karyotypes in stocks from 10 different regions of Indonesia has shown great variability in their chromosome profiles, and that the similarities in the banding patterns are related to the stock origin. Within these banding patterns there were bands that were sufficiently stable to enable them to be used to identify stocks. The aim of the present study was to develop a method to produce a chromosome probe that can identify related *T. evansi* stocks from different areas.

4.7.2. MATERIALS AND METHODS

4.7.2.1. Trypanosomes

Agarose blocks of *T. evansi* DNA, TREU 2225, were separated by TAFE under conditions reported to resolve the chromosomes in the 50-500 kb size range. The running conditions used have been described in Section 4.5.2.2. Gels were prepared using 1% LE agarose in most cases. When the DNA elution was carried out using GELase, treatment gels were prepared using the 1.3 % LMP. Five identical *T. evansi* DNA samples from the same stock were subjected to TAFE to provide sufficient material for chromosomal DNA extraction from the agarose gel. After the TAFE run was finished the gel was stained with ethidium bromide (0.5 μg/ml) and then destained for 30 minutes in distilled water. The gel was visualised on the UV transilluminator (302 nm) and the bands sizes estimated relative to those of the lambda DNA concatemer size standard. The 200 kb band in each track of the gel was cut out of the gel using a sterile surgical blade and transferred into a 1.5 ml microcentrifuge tube.

4.7.2.2. Elution of DNA from Agarose Gels using GeneClean II® Kit

DNA was extracted from the agarose matrix by following the protocol described by the manufacturer of GeneClean II® kit (Bio 101 Inc. La Jolla). The volume of the gel slice was first determined by weight (1 g agarose = 1 ml) and then 4.5 volumes of sodium iodide (NaI) stock solution and 0.5 volumes of TBE modifier was added into the tube containing the gel slice. The eppendorf tube was then incubated in a 55^{0} C waterbath for 5 minutes or until all the agarose had dissolved. The Glassmilk preparation was first vortexed to resuspend the insoluble silica matrix and then 20 μl of the suspension was added to the tube containing the mixture of NaI, molten agarose and DNA. The tube and contents was then gently mixed and incubated at room temperature for 5 minutes to allow binding of the DNA to the silica matrix. The sample was mixed by flicking every 2 minutes, to ensure that the Glassmilk stayed suspended. The silica matrix containing the bound DNA was pelleted by centrifugation at 14000 rpm for 5 seconds in a microcentrifuge and the NaI solution was removed and saved in a separate tube in case the DNA did not bind to the silica matrix. The pellet was washed three times by diffusion with the New Wash solution provided in the kit. In each wash, the New Wash solution was removed after 5 minutes and replaced with a fresh aliquot. The DNA was eluted from the Glassmilk pellet by resuspending the washed pellet with 20 μl TE buffer pH 7.4. The resuspension was carried out as gently as possible using a large bore pipette tip to prevent shearing of the DNA. The tube was incubated at 55^{0} C for 3 minutes and then centrifuged at 14000 rpm for 30 seconds. The supernatant, which contained the eluted DNA was carefully removed and placed in a clean, sterile tube. The pellet was then

eluted for a second time with a further 10 µl TE buffer. DNA eluted by this technique was either used immediately or stored at -20^0 C. The presence of DNA in the eluate was confirmed by a conventional agarose gel electrophoresis and ethidium bromide staining. The DNA sample quantitation was carried out by an ethidium bromide quantitation method (Sambrook, Fritsch and Maniatis, 1989).

4.7.2.3. Elution of Chromosomal DNA by Electroelution

One of the agarose slices (Section 4.7.2.1) containing the 200 kb DNA fragment of TREU 2225 was subjected to electro-elution in a Model 422 Electro-eluter (BioRad) in an attempt to recover the chromosomal DNA in solution. Prior to the electroelution the DNA band slice was digested overnight at 37^0 C with 50 Units of *Eco*R I (Sambrook *et al.*, 1989) to reduce the size of DNA fragments and thus facilitate elution. The gel slice was first washed with 50 volumes of TE buffer (pH 7.4) for 30 minutes at room temperature and then transferred into a 1.5 ml microcentrifuge tube. The 1x restriction enzyme buffer provided with the enzyme was added at 10 volumes and incubated for 30 minutes at 4^{0}C. After incubation the buffer was removed and 3 volumes of fresh 1x restriction enzyme buffer added, followed by a further 50 units of *Eco*R I. The reaction was then incubated at 37^0 C for 16 hours. At the end of the incubation the agarose block was soaked in 50 volumes of TE buffer (pH 7.4) for 1 hour at 4^{0}C to allow the salts in the restriction enzyme buffer to diffuse from the agarose sliced blocks. The *Eco*R I digested 200 kb fragment was then subjected to electroelution according to the protocol recommended by the manufacturer (BioRad). Prior to use, the 12 kda membrane caps were soaked for at least 1 hour at 60^0 C in DNA elution buffer (40 mM Tris; 20 mM Glacial acetic acid; 1 mM EDTA pH 8.0; 0.1% SDS). Gloves were worn when handling the membrane caps to prevent the dialysis membrane from becoming contaminated. The Model 422 Electro eluter was then assembled according to the manufacturer's instructions (BioRad, UK). The elution of DNA from the gel slice was carried out at ~10 mA/ constant current per tube for 1 hour. The eluate was removed and the electroelution continued for a further 2, 5 and 20 hours. The eluate was removed at the end of each elution time period.

At the end of each electro-elution run the polarity was reversed for approximately 1 minute to remove the DNA from the dialysis membrane. The electro-eluter module was taken out of the buffer tank, with the glass tubes attached, to a sink. The stopper was removed from the upper buffer chamber and the buffer was allowed to drain out. Using a plastic pipette, the buffer in the tube was removed down to the level of the frit and discarded. The liquid below the frit should not be disturbed or shaken up during this process. The silicone adaptor was then removed together with the membrane cap from the bottom of the glass tube. The buffer level was kept at the top of the membrane cap. Using a clean plastic pipette, the liquid

remaining in the membrane cap (approximately 400 µl) was pipetted into a microfuge tube. The membrane cap was rinsed with 200 µl of fresh elution buffer. The liquid was added to the centrifuge tube that contains the eluted DNA. The total volume collected was approximately 600 µl. The eluted DNA had to be purified for further use. The same membrane caps were reused because the elution was carried out for the same fragment. The presence of DNA after electro-elution was detected by agarose gel electrophoresis and ethidium bromide staining. The eluted DNA was purified by ethanol precipitation as described by Sambrook *et al.* (1989) (Section 4.7.2.5).

4.7.2.4. Elution of Chromosomal DNA using GELase®

The 200 kb DNA fragment of TREU 2225 (Section 4.7.2.3) was treated with GELase® (Epicentre Technologies) to recover the chromosome sized DNA from the agarose. The TAFE was carried out in a 1.3% LMP agarose using normal TAFE running conditions. The digestion of the gel slice with GELase® was carried out according to the manufacturer's recommendations. The gel slice containing 200 kb band was weighed to determine its volume (1 g agarose = 1 ml) and then soaked in 3 volumes of 1x GELase® buffer (40 mM Bis-Tris pH 6.0 and 40 mM NaCl) for 1 hr. The buffer was removed from the tube by pipetting and then the gel was melted at 70^0 C for 20 minutes. The molten agarose was equilibrated to 45^0 C for 10 minutes and an appropriate amount of GELase® (1 unit per 600 mg of 1% LMP agarose gel) added. The tube and contents were incubated at 45^0 C for 1hr. The DNA from the agarose/GELase mixture was purified by ethanol precipitation (Sambrook *et al.*, 1989) (Section 4.7.2.5).

4.7.2.5. Ethanol Precipitation of DNA (Sambrook et al.*, 1989)*

The ice-cold ethanol was added at 2.5 volumes to the eluted DNA and incubated at -20^0 C overnight. The DNA pellet was obtained after centrifugation at 14000 rpm for 5 minutes and removal of the supernatant. The DNA pellet was resuspended in 100 µl of TE buffer pH 7.4 and the ethanol precipitation repeated. The final pellet of DNA was rinsed with 70% ethanol, centrifuged and the ethanol was discarded. The DNA pellet was then air dried and resuspended in 10 µl of TE buffer and stored at 4^0 C.

4.7.2.6. Quantitation of DNA by Ethidium Bromide

The presence of DNA in eluted samples was determined by mixing 1 µl of each sample with 1 µl of ethidium bromide (1µg/ml) on a 4 cm square piece of parafilm marked out into two rows. The first row was used for the standard DNA dilution series and the second row for

the samples under test and the no-DNA control (ethidium bromide and H_2O only). Serial dilutions from 0.75, 1.5, 3.1, 6.2, 12.5, 25, 50 and 1 00 μg/ml of a DNA standard made from sonicated salmon sperm DNA (Pharmacia) were added (1μl each dilution) to the ethidium bromide in the first row. The parafilm sheet containing these samples was examined on a 254 UV transilluminator. Samples containing DNA gave the usual pinkish fluorescence. The fluorescence intensity of each test sample was compared to the intensities of the DNA standard to estimate the test DNA concentration.

4.7.3. RESULTS

4.7.3.1. Chromosomal DNA Extraction using GeneClean II Kit

The eluate prepared from the 200 kb band cut from a 1% agarose TAFE gel and processed using the GeneClean II kit did not show any bands in the agarose gel. The eluate was further tested for DNA using an ethidium bromide DNA detection method. The sample fluorescence was compared to that of the standard DNA dilutions. Very weak fluorescence was observed in the test samples compared to the lowest dilution (0.75 μg/ml) of the standard DNA indicating very little DNA contained in the GeneClean eluate.

4.7.3.2. Chromosomal DNA Extraction by Electroelution

The eluate prepared from the DNA from the 200 kb band slice and subjected to electroelution contained salt granules in the sediment after ethanol precipitation. This salt was always seen in all of the electroelution products and dissolved on the addition of TE buffer. The presence of DNA was not detected in ethidium bromide stained agarose gel in the ethanol precipitated eluate obtained after 1, 2 and 5 hours electroelution time. However, the eluted DNA sample obtained after 20 hours-elution showed fluorescence in the agarose electrophoresis sample well indicating the presence of a high molecular weight DNA.

4.7.3.3. Chromosomal DNA Extraction using GELase®

The presence of DNA after GELase® treatment in the 200 kb band slice was not detected in the ethidium bromide stained agarose gel. The fluorescence of the undiluted DNA samples recovered by GELase® and ethanol precipitated was then compared with that of the standard DNA dilutions to confirm the presence of DNA in the eluate. None of the test samples showed any fluorescence suggesting that there was very little, if any, DNA present in the sample.

4.7.4. DISCUSSION

The presence of the chromosomal DNA eluted from a band in PFGE gel using the GeneClean II kit was not detected by agarose gel electrophoresis. This may have been due to an insufficient amount of DNA run in the gel or loss of DNA during the GeneClean elution procedure. Fluorescence was however, detected in the agarose electrophoresis sample well containing Glassmilk, indicating that the DNA was still bound to the Glassmilk. The absence of DNA after GeneClean extraction might be because the DNA was not completely released from the 1% LE agarose due to the fact that the LE agarose was not completely melted. The gel slice did not melt although it was cut into small pieces and put in the 70^0 C waterbath for 30 minutes. The use of 1.3% LMP agarose in the TAFE was also carried out and the band slice was processed using the GeneClean kit. The presence of DNA was not detected in the agarose gel electrophoresis. These results suggest that the GeneClean procedure was not suitable for eluting large molecular weight DNA.

The presence of DNA in the eluate after electroelution and ethanol precipitation was not detected in the agarose gel electrophoresis. This might be due to the large volume of elution buffer used for diluting the DNA or a lot of DNA remains in the dialysis membrane (Birren and Lai, 1993). Some DNA will be irreversibly bound to the dialysis membrane, even if the polarity is reversed at the end of electroelution and removal of the DNA from the electroelution membrane will cause damage of the DNA (Birren and Lai, 1993). Typical electroelution yields for DNA are claimed to be between 70% and 90% (BioRad, UK). In the present study the DNA contained in the 200 kb band slice was first restriction enzyme digested by *Eco*RI in an attempt to reduce the size of DNA. The absence of DNA in the eluate after electroelution might be caused by two different reasons. First, the enzyme might not have cut the DNA in the 200 kb band slice causing large molecular weight DNA to remain in the dialysis membrane. Secondly, the enzyme might have cut the 200 kb DNA into small pieces with molecular sizes less than the 12 kb membrane cut-off which consequently passed through the dialysis membrane and were lost in the electroelution buffer.

The third method which attempted to recover DNA from a chromosomal band slice using GELase enzyme digestion was unsuccessful which might be due to the insufficient amount of GELase used to digest the 1.3% LMP agarose. It was recommended to use 1 unit of GELase to digest 600 mg of molten 1% LMP agarose. Higher concentration (1.3%) of LMP agarose used in the present study should require higher concentration of GELase to yield a complete digestion of the LMP agarose used. The GELase method is claimed to allow recovery of intact DNA of 100 bp to Megabase chromosomal DNA (Epicentre Technologies, UK).

Several approaches were carried out to recover the DNA from agarose gel, but the results were unsatisfactory. Unsuccessful results might be due to the: 1) lack of DNA in the gel slice; 2) incomplete digestion by the restriction enzymes; or 3) contamination which might inhibit digestion or elution.

4.8. CHROMOSOME LOCATIONS OF HOUSEKEEPING GENES IN *TRYPANOSOMA EVANSI* STOCKS COLLECTED FROM INDONESIA

4.8.1. INTRODUCTION

The molecular karyotype of *T. evansi* stocks collected in Indonesia, determined by analysis of chromosome-sized DNA bands separated by the TAFE system, showed a high degree of variability in their chromosomal profiles. It was reported that in *T. brucei* the gene of triosephosphate isomerase (TIM) is located in a high molecular weight chromosome of approximately 5.7 Mb (Gottesdiener *et al.*, 1990). The gene marker for glyceraldehyde phosphate dehydrogenase (GAPDH) hybridised to two Megabase size bands in *T. brucei* (Gibson and Borst, 1986; Gottesdiener *et al.*, 1990) and several bands in *T. cruzi* (Gibson and Miles, 1986) indicating that there are multiple GAPDH genes scattered across several chromosomes (Gibson and Miles, 1986). Furthermore it was suggested that the GAPDH genes are located separately from other genes such as TIM, Phosphoglycerate kinase (PGK) and tubulin (Gibson and Borst, 1986). In *T. brucei* the Aldolase (ALD) gene is located at the DNA that remained in the gel slot in (Gibson and Borst, 1986; Gibson and Garside, 1991;), however in *T. cruzi* this gene probe hybridised in the 800 kb and 1,000 kb band size (Gibson and Miles, 1986). In *T. brucei* the glucosephosphate isomerase (PGI) and Tubulin genes are located on the same chromosome bands (Gibson and Borst, 1986), that is, at 1500 kb and 3000 kb (Gottesdiener *et al.*, 1990). Gibson and Borst (1986) suggested that the tubulin and PGK genes are linked on the same chromosomes.

The resolution by PFGE had facilitated further studies on the chromosomal organisations in trypanosomes. Six specific gene probes, Phospholipase C (PLC), Cysteine Proteinase (CP), *T. brucei* rRNA coding region (λ104), *T. brucei* tubulin (β tubulin), Aldolase (ALD) and *T. brucei* glucose-6-phosphate isomerase (PGI) were use to hybridise the chromosomal DNA blots of TAFE gel transferred on to nylon membrane. This study was carried out to provide further information on the karyotype variability of *T. evansi* stocks with respect to chromosomal localisation of each gene marker.

4.8.2. MATERIALS AND METHODS

4.8.2.1. Trypanosoma evansi *Stocks and PFGE Conditions*

Fourteen *T. evansi* stocks representative of 7 karyotype groups; one *T. brucei* and one *T. congolense* stocks were used in this study (**Table 4.6**). Agarose embedded DNA from the selected stocks was subjected to a TAFE using the conditions to resolve bands between 50-900 kb (Section 4.5.2.2). After ethidium bromide staining (0.5 μg/ml) and photography, the PFGE gel was transferred onto a glass plate and subjected to Southern blot using capillary transfer.

4.8.2.2. Probes

The probes used in this study were obtained from Drs. G. Hide and W. Gibson. Random primed DNA labelling using digoxigenin-dUTP from Boehringer Mannheim (GmbH, Biochemica) was used to label the probes. The amount of each probe labelled was suggested by Drs Hide and Gibson. The probes, Phospholipase C (PLC); Cysteine Proteinase (CP) and *T. brucei* ribosomal RNA coding region (λ 104) were each used at a volume of 5 μl (~12.5 ng) per 20 μl final reaction volume. The *T. brucei* tubulin (β tubulin) (Thomashow, Milhausen, Rutter *et al.*, 1983) and aldolase (ALD) (Marchand, Poliszczak, Gibson *et al.*, 1988) probes were supplied by Dr. Gibson at a concentration of 50 ng/μl for each probe and were used at ~10 ng per 20μl labelling reaction. A sequence of the *T. brucei* glucose-6-phosphate isomerase gene (Marchand *et al.*, 1988) was PCR-amplified and labelled. The labelled probe was used to hybridise with the trypanosome stocks at a volume of 5 μl (~72.5 ng).

4.8.2.3. Digoxigenin DNA Labelling

Random-primed labelling using Digoxigenin-11-dUTP, the DIG DNA Labelling Kit (Boehringer Mannheim, GmbH, Biochemica) was used to label the DNA probes. Before labelling the DNA was denatured at 95^0 C for 10 min followed by chilling quickly on ice. For each reaction the following reagents were added into a microfuge tube on ice: 1μg freshly denatured DNA, 2 μl hexanucleotide mix, 2 μl dNTPs labelling mix, sterile H_2O was added up to 19 μl volume and 1 μl Klenow enzyme. The reaction was incubated overnight at 37^0 C and then stopped by adding 2 μl of 0.2 M EDTA solution, pH 8.0.

4.8.2.4. Southern Blot Transfer

The Southern blot by capillary transfer was carried out according to the method initially described by Southern (1975) and modified by Birren and Lai (1993). Because of the large DNA size, the DNA was depurinated by soaking the gel in 0.25 N HCl for 30 min and then rinsing it briefly with deionized water. The DNA was then denatured by soaking the gel in a solution containing 0.4 N NaOH, 0.6 M NaCl for 30 minutes. The depurinated DNA was then transferred onto a nylon membrane (Hybond, Amersham) using a wick in the presence of 20x SSC, pH 7.0 (3.0 M NaCl, 0.30 M Trisodium citrate). A diagram showing the standard assembly for upward capillary transfer of DNA from agarose gel onto a nylon membrane filter is presented in **Figure 4.22**.

The nylon membrane was cut about 1 mm larger than the gel size in both dimensions. The membrane was floated on the surface of a dish of deionised water until completely wet. The membrane was then immersed in transfer solution (20x SSC) for at least 5 minutes. The denatured DNA was transferred from the gel to the membrane by capillary action. The 3 MM filter paper (3 sheets) was cut bigger than the gel size so that the two sides of the paper covered the support and dipped in the transfer buffer. After removal from the denaturation solution the gel was inverted so that its underside was now uppermost. The gel was placed on a support which had been placed in a container containing the transfer buffer (20 x SSC) and centered on the wet 3 MM papers, which had been cut so that the sides of the filter paper dipped into the transfer solution. Air bubbles between 3MM paper and the gel were removed by smoothing the gel surface. The wet nylon membrane was placed on top of and aligned with the gel. The nylon membrane was not moved after it had been applied to the surface of the gel. Air bubbles between the filter and the gel were removed by smoothing the surface using a glass rod. Two pieces of 3MM paper cut to exactly the same size as the gel were soaked in 20 x SSC and placed on top of the wet nylon filter. After removing any air bubbles, a 12 cm high stack of paper towels was piled on the 3MM papers. A glass plate was placed on top of the stack and a 500 g weight placed on top of the stack. The capillary transfer of DNA was allowed to proceeded overnight. After the DNA was transferred, the paper towels and the 3MM papers above the gel were removed. The positions of the sample slots on the filter were marked with a pen and then the gel and the nylon filter were turned over and laid gel side up, on a dry sheet of 3MM paper. The gel was peeled from the membrane and the gel was stained for 45 minutes in ethidium bromide solution and examined by UV transillumination to assess the efficiency of the DNA transfer. The membrane was rinsed twice for 5 minutes each, in 2 x SSC, blotted dry and covered in cling film before fixing. The DNA was fixed by exposing the filter membrane to UV in the GS Gene Linker® (BioRad) using the program C2 (50 mJ) when the membrane was dry or C3 (150 mJ) when the membrane was damp.

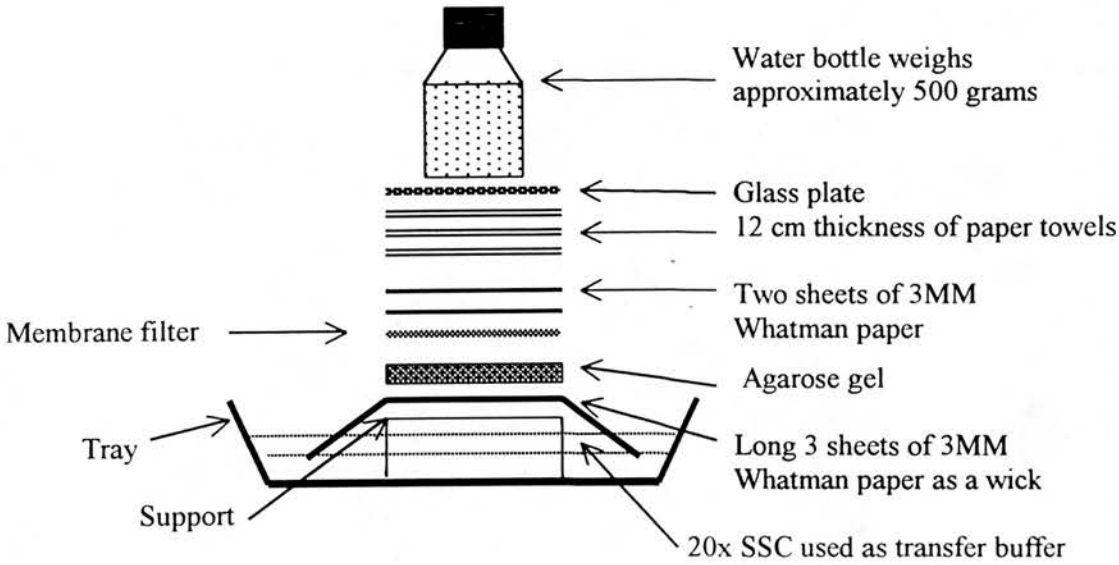


Figure 4.22. Standard assembly for upward capillary transfer of DNA from agarose gel onto a nylon membrane filter.

4.8.2.5. Southern Blot Hybridisation

After washes and UV fixation, the membranes were pre-hybridised to block non-specific nucleic acid binding sites on the membrane. The membranes were then put into hybridisation bottles (15 x 3.5 cm), and 6 ml of hybridisation buffer (5x SSC, 50% formamide, 0.1% N-laurylsarcosine, 0.02% w/v SDS, 2% blocking reagent) was added into each tube. The hybridisation buffer was prepared by warming slowly in a microwave for 60 seconds, using low power set (power 3) and shaking every 10 seconds to dissolve the blocking reagent. Incubation was carried out at 42^0 C for at least 1 hour in a Hybaid rotary hybridisation oven. The hybridisation solution was discarded and replaced with hybridisation buffer containing the denatured, labelled probe.

The labelled probe was denatured in boiling water for 10 minutes, and then immediately chilled in iced water. The denatured probe was then added to 6 ml of hybridisation solution previously warmed to 42^0 C. When required the hybridisation solution containing the labelled probe can be stored frozen at -20^0 C and re-used. Prior to reuse, the probe has to be denatured at 68^0 C for 10 minutes.

4.8.2.6. Chemiluminescent Detection using Digoxigenin dUTP (Boehringer Mannheim)

Before the detection, the membranes were washed twice for 5 minutes each wash in 2x SSC, 0.1% SDS at room temperature and then twice for 15 minutes each wash in 0.1x SSC, 0.1% SDS at 68^0 C under constant agitation.

The chemiluminescent detection involves three steps. After hybridisation and stringency washes, the membranes were rinsed briefly (2 minutes) in the washing buffer (0.1 M Tris, 0.15 M NaCl, pH 8.0, 0.3% v/v Tween20). A blocking step was carried out by incubating the membrane in the blocking buffer (0.1 M Tris, 0.15 NaCl, pH 8.0; 1% blocking reagent) for 30 minutes; when the β tubulin gene probe used, the blocking step was increased to 1 hour.

The membrane was incubated with the Anti-DIG-alkaline phosphatase conjugate diluted in 1:10,000 (75 mU/ml) with DIG blocking buffer for 30 minutes. The antibody solution was discarded and the membrane washed 2 x 15 minutes each wash with DIG washing buffer. The membrane was equilibrated for 2 minutes in DIG detection buffer (0.1 M Tris, 0.1 M NaCl, 50 mM $MgCl_2$). The Lumigen™ PPD [4-methoxy-4-(3-phosphate-phenyl)-spiro (1,2-dioxetane-3,2-adamantane) disodium salt] was diluted 1:100 in DIG detection buffer and added using a sterile pipette on to the membrane which had been placed between two sheets of acetate. Approximately 0.5 ml of the detection buffer containing the Lumigen™

PPD was added per 100 cm^2, evenly spread over the membrane by rocking gently. Air bubbles were removed by wiping the top of the sheet with a tissue. The membrane was incubated in the Lumigen™ PPD solution for 5 minutes. After the incubation, the excess liquid was removed by blotting the membrane briefly on Whatman 3MM paper. The membrane was then sealed in a cling film sheet and further incubated at 37^0 C for 15 minutes. The membrane was exposed to an X-ray film for at least 30 minutes; in some cases it had to be exposed overnight. The film was photographed using Polaroid MP-4 Land Camera, without filter, and Type 55 Polaroid film. The f stop was set at 11 with 1/28 seconds exposure time and 45 seconds developing time.

4.8.2.7. Stripping and Reprobing of DNA Blots

Stripping of DNA blots for reuse in several hybridisation experiments was carried out according Dubitsky, Brown and Brandwein (1992). First, the membrane was washed twice for 30 min each wash in water containing Proteinase K 0.5-1 mg/ml and SDS 0.1-0.2% w/v at approximately 68^0 C. The second wash was carried out twice for 5 min each wash in 2x SSC, then in 0.1x SDS. To strip the probe, the membrane was then washed twice for 30 min each wash in 50% w/v formamide, 10 mM NaH_2PO_4, pH 6.5 at 68^0 C and rinsed in 2x SSC. Re-probing for the stripped membrane was carried out with pre-hybridisation and hybridisation steps as described above. The membrane can be stored wet after stripping in 2x SSC in a sealed plastic bag.

4.8.2.8. Production of PGI Gene Probe by PCR Amplification

4.8.2.8.1. Isolation of genomic DNA from *T. evansi* stock TREU 2311

High molecular weight DNA was extracted from TREU 2311 according to the method described by Sambrook *et al.* (1989) for isolating. Trypanosomes separated by DE52 chromatography (Section 3.2.2) were resuspended in 1 ml TE buffer (10 mM Tris, 1 mM EDTA) and then transferred to sterile 50 ml centrifuge tubes. Ten ml of extraction buffer (10 mM TrisCl (pH 8.0), 0.1 M EDTA (pH 8.0), 20 μg/ml RNAse, 0.5% SDS) was added and the mixture was incubated for 1 hour at 37^0 C. Proteinase K (0.5 mg/ml) was added to the mixture to give a final concentration of 100 μg/ml. The enzyme was mixed gently into the viscous solution using a glass rod or a sealed Pasteur pipette. Protein digestion was carried out by incubating the solution for 3 hours at 50^0 C. The centrifuge tube containing the digested trypanosomes was removed from the waterbath and the solution cooled to room temperature.

The trypanosomal DNA was extracted by phenol extraction (Sambrook *et al.*, 1989). Prior to use the phenol was equilibrated according to the method described by Sambrook *et al.* (1989). Liquified phenol stored at -20^0 C was removed from the freezer, warmed to room temperature and then melted at 68^0 C in a waterbath. The 8-hydroxyquinoline was added to a final concentration of 0.1%. An equal volume of 0.5 M TrisCl buffer pH 8.0 was added to the melted phenol and stirred for 15 minutes. The upper aqueous phase was then aspirated. An equal volume of 0.1 M TrisCl buffer pH 8.0 was added to the phenol and stirred for 15 minutes. The upper aqueous phase was removed as before. The equilibration was repeated until the pH of the phenolic phase was >7.8 (measure with the pH paper). The 0.1 M TrisCl buffer pH 8.0 containing 0.2% β-mercaptoethanol was then added at 0.1 volume to the equilibrated phenol and stored at 4^0 C until used.

An equal volume of equilibrated phenol was added and mixed gently by inversion for 10 minutes. The two phases were separated by centrifugation at 5,000 g for 15 minutes at room temperature. The viscous aqueous phase was then transferred, using wide bore pipette tips, into a clean centrifuge tube. The phenol extraction was repeated twice by adding an equal amount of TE buffer and gently mixing as described above. The aqueous solutions were pooled and the DNA extracted by ethanol precipitation (Section 4.7.2.5).

4.8.2.8.2. PCR amplification of PGI

The primers used for the amplification of Glucose-6-phosphate isomerase (PGI) gene sequence were described by Marchand *et al.* (1988). The primers sequences were synthesised by Cruachem: PG1: 5'-ACGACAGAGAACCGCCCAGTC-3' and PG2: 5'-CTTCTCATCGATGCCGCGCGA-3'. Primers were stored at a concentration of 2 μM in sterile 18.2 MΩ water and used at a concentration of 0.2 μM for the PCR assay.

The PCR reaction contains 10 mM Tris-HCl buffer pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTPs; 0.2 μM of each primer; 2.5 units *Taq* DNA polymerase (Boehringer Mannheim; GmbH, Biochemica) and 10 ng of trypanosomal genomic DNA. The PCR was carried out on a Hybaid thermal cycler programmed for 30 cycles; at 94^0 C for 1 minute for denaturation; 55^0 C for 2 minutes for annealing and 72^0 C for 2 minutes for extension.

The PCR amplification products were detected by agarose gel electrophoresis using an 0.8% gel in the presence of Loening buffer (35.8 mM Tris, 39.2 mM Sodium dihydrogen phosphate, 5 mM EDTA disodium dihydrate).

The PCR product samples were used without purification, and mixed (5:1) with a gel loading dye solution (Amresco, Solon, Ohio). The sample-dye mixture (5 μl) and 3 μl of the 1 kb standard marker (Gibco, BRL) were slowly loaded into the slots. The electrophoresis was

carried out at 60 Volts and stopped when the bromophenol blue had migrated an appropriate distance through the gel (usually the gel was run for 30-45 minutes). After removing the gel from the tank, it was examined under UV transillumination and photographed. The band sizes were calculated by comparison to the standard marker.

4.8.2.8.3. Labelling of the PGI amplification product

The PCR amplification product was labelled, without prior purification, using DIG DNA Labelling Kit (Section 4.8.2.3). The amount of DNA was estimated by ethidium bromide quantitation (Sambrook *et al.*, 1989) as described in Section 4.7.2.6.

4.8.3. RESULTS

4.8.3.1. PGI gene Probe Production

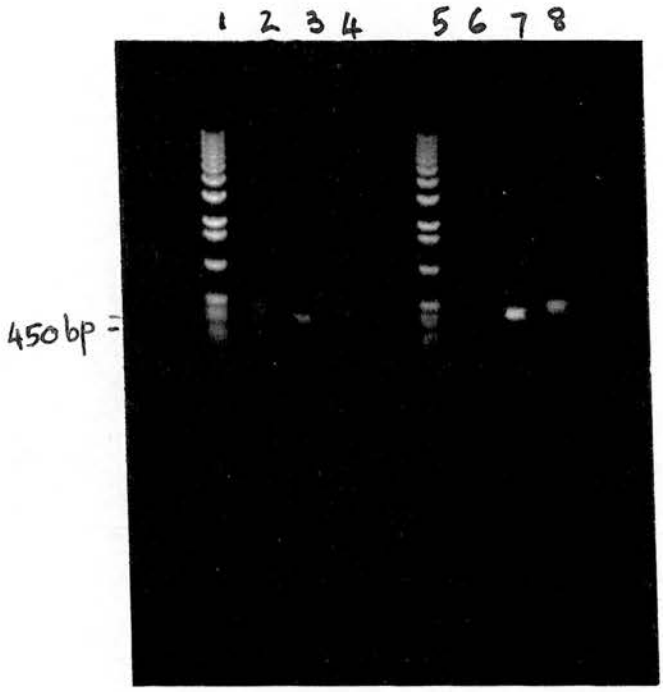
The TREU 2311 amplification product using the PGI primers yielded a band of approximately 450 bp (**Figure 4.23**). A band of larger than 12 kb was detected in lanes 2 and 6 that contained 10 ng genomic *T. evansi* DNA. The amplification product of the PGI gene sequence yielded a band of approximately 450 bp (lane 3 and 7); the labelled product (lane 4 and 8) showed a larger (~500 bp) band than that of the unlabelled product.

The ethidium bromide quantitation (**Figure 4.24**) for PGI gene probe was carried out to estimate the amount of DNA contained in the unlabelled and labelled PCR products. The spot containing 10 ng *T. evansi* genomic DNA showed similar fluorescence intensity with that of the 12.5 ng/μl DNA standard. The unlabelled PGI gene amplification product showed similar fluorescence intensity to the 3 ng/μl standard DNA. The labelled probe, however, showed higher fluorescence intensity (12.5 ng/μl) than that of the unlabelled probe.

4.8.3.2. Chromosomal Location of Six Genetic Markers Trypanosomes

The karyotype patterns of trypanosome stocks used in the present study as shown in **Figure 4.25** have been discussed in Section 4.3.3.4. The trypanosomal chromosomes separated in the PFGE agarose gel were then transferred on to a nylon membrane and hybridised with the gene probes described in Section 4.8.2.2.

Figure 4.23. The amplification product using Glucose-6-phosphate isomerase primers showed a band at ~450 bp .


1 2 3 4 5 6 7 8
450bp

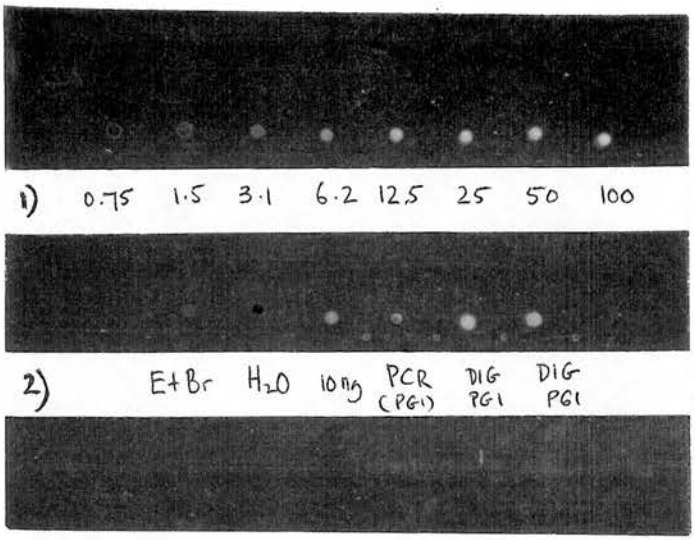


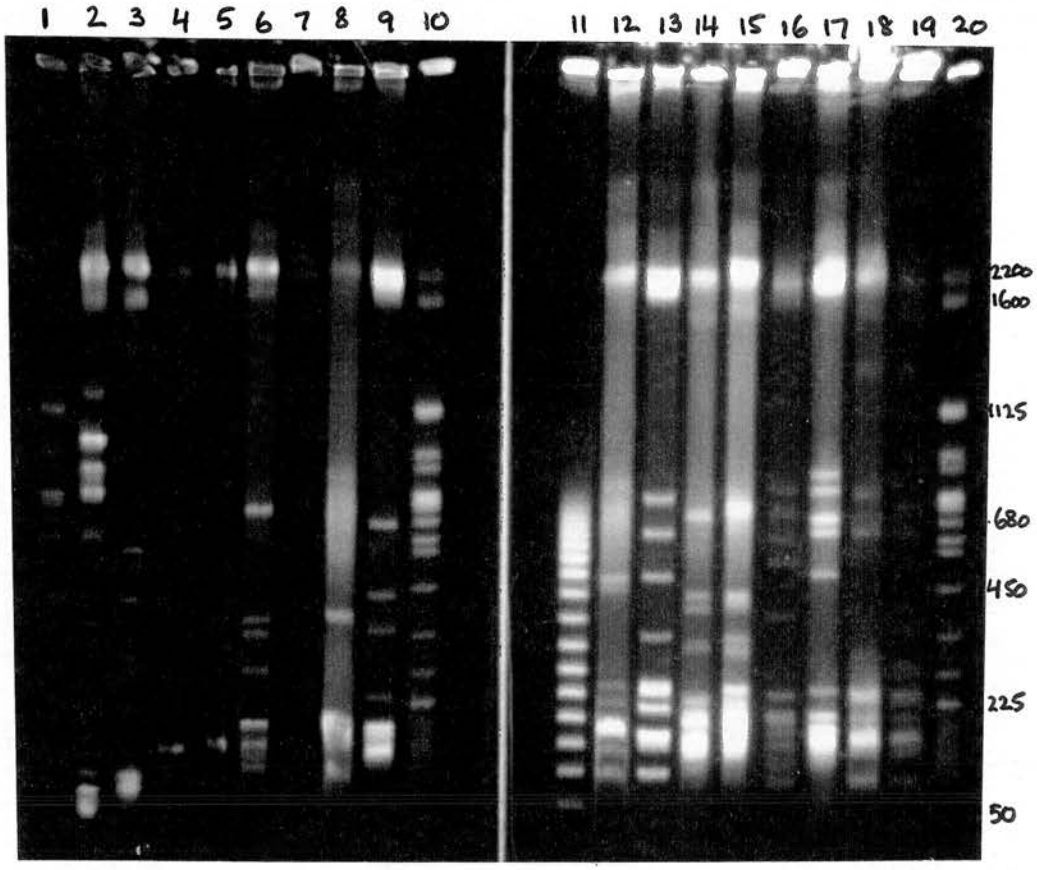
Figure 4.24. Ethidium bromide quantification of PGI probe.

Row 1: Serial dilutions of DNA standard (0.75, 15, 3.1, 6.2, 12.5, 25, 50, 100 μg/ml).

Row 2: EtBr = Ethidium bromide
H_2O = Water, no DNA
10 ng = 10 ng of genomic DNA
PCR (PGI) = unlabelled PCR product with PGI primers
DIG PGI = labelled PCR product with PGI primers

Figure 4.25. Chromosomal banding patterns of *Trypanosoma evansi, T. brucei* and *T. congolense* stocks used for hybridisation with six different genetic markers separated by Transverse Alternating Field Electrophoresis using conditions to resolve DNA in the 50-900 kb size range.

Tracks 1, 10, 20: *Saccharomyces cerevisiae* size standard marker;
Track11: lambda DNA concatemer size standard
Track 2: TREU 2193/*T. congolense*
Track 3: TREU 2177/*T. brucei*
Track 4: BAKIT 503 (group 2)
Track 5: BAKIT 427 (group 2)
Track 6: BAKIT 399 (group 2)
Track 7: BAKIT 500 (group 2)
Track 8: BAKIT 409 (group 1.6)
Track 9: BAKIT 461 (group 1.6)
Track12: BAKIT 423 (group 1.5)
Track13: BAKIT 467 (group 1.5)
Track14: BAKIT 382 (group 1.4)
Track15: BAKIT 401 (group 1.4)
Track16: BAKIT 508 (group 1.2)
Track17: BAKIT 100 (group 1.3)
Track18: BAKIT 505 (group 1.1)
Track19: BAKIT 134 (group 1.1)


1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
2200
1600
1125
680
450
225
50

Southern blot hybridisation of the self made PGI gene probe is shown in **Figure 4.26**. The probe did not hybridise with any DNA in the size markers used (tracks 1, 10, 11 and 20). In all stocks the probe hybridised with DNA in the sample well. In *T. congolense* the PGI probe also hybridised with the minichromosomal DNA smaller than 50 kb; hybridisation was not observed in DNA either in the megabase size or in the intermediate sizes (lane 2, **Figure 4.26**). Hybridisation was detected in *T. brucei* all of the chromosomal bands in large (1.6 and 2.2 Mb), intermediate (200-570 kb) and small (50-150 kb) chromosomes (Track 3, **Figure 4.26**). In *T. evansi* the PGI probe hybridised more strongly in most of the intermediate and small chromosomal bands and the DNA that remained in the sample wells than in large chromosomes (Tracks 4-9 and 12-19, **Figure 4.26**). It was also noted that the PGI gene probe was not hybridised in all intermediate and small chromosomes of *T. evansi* as observed in BAKIT 508 (Track 16, **Figure 4.26**) and BAKIT 100 (Track 17, **Figure 4.26**).

The hybridisation patterns of *T. brucei* tubulin gene (β tubulin) probe in the stocks studied are presented in **Figure 4.27**. Strong hybridisation signals were detected in the sample wells in all stocks studied. In *T. congolense* the tubulin probe hybridised weakly in large DNA of approximately 2.2 Mb (Track 2, **Figure 4.27**). Hybridisation in *T. brucei* was also found in large DNA (~2.2 Mb) and two bands in the compression zone (Track 3, **Figure 4.27**). Hybridisation of the *T. brucei* β tubulin probe showed different patterns among the *T. evansi* stocks subjected to the study. The hybridisation signals of β tubulin probe in each stock were presented in **Table 4.15**. It was observed that the hybridisation patterns of β tubulin probe in *T. evansi* stocks BAKIT 500, 423, 467 and 100 were similar to that of *T. brucei*. It was also noted that the β tubulin hybridisation patterns in *T. evansi* corresponded with karyotype group previously assigned in Section 4.3.3.1. The β tubulin hybridisation pattern of *T. congolense* was different from those shown by *T. brucei* and *T. evansi* by the absence of the hybridisation signals both chromosomal bands in the compression zone and 1.6 Mb size.

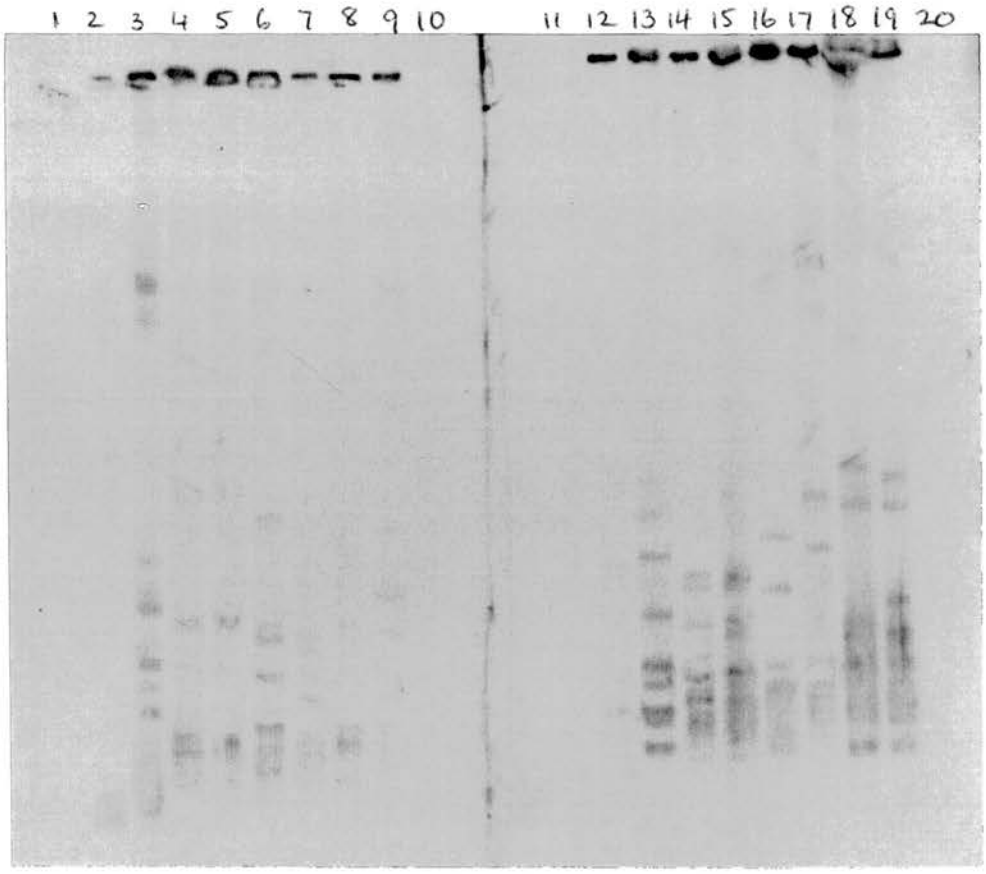


Figure 4.26. Southern blot hybridisation of *T. evansi*, *T. brucei* and *T. congolense* stocks separated by Transverse Alternating Field Electrophoresis with Glucose 6 phosphate isomerase (PGI) gene probe.

Tracks 1, 10, 20: *Saccharomyces cerevisiae* size standard marker;
Track11: lambda DNA concatemer size standard
Track 2: TREU 2193/*T. congolense*
Track 3: TREU 2177/*T. brucei*
Track 4: BAKIT 503 (group 2)
Track 5: BAKIT 427 (group 2)
Track 6: BAKIT 399 (group 2)
Track 7: BAKIT 500 (group 2)
Track 8: BAKIT 409 (group 1.6)
Track 9: BAKIT 461 (group 1.6)
Track12: BAKIT 423 (group 1.5)
Track13: BAKIT 467 (group 1.5)
Track14: BAKIT 382 (group 1.4)
Track15: BAKIT 401 (group 1.4)
Track16: BAKIT 508 (group 1.2)
Track17: BAKIT 100 (group 1.3)
Track18: BAKIT 505 (group 1.1)
Track19: BAKIT 134 (group 1.1)

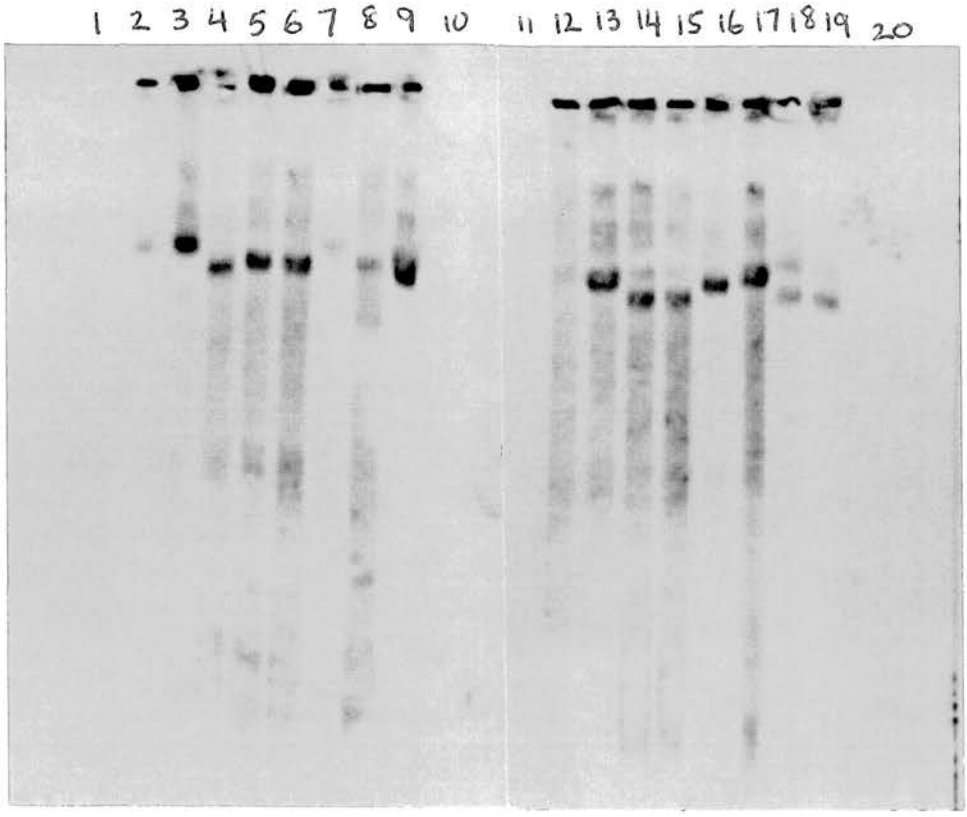


Figure 4.27. Southern blot hybridisation of *T. evansi*, *T. brucei* and *T. congolense* stocks separated by Transverse Alternating Field Electrophoresis with *T. brucei* β-tubulin gene probe.

Tracks 1, 10, 20: *Saccharomyces cerevisiae* size standard marker;
Track11: lambda DNA concatemer size standard
Track 2: TREU 2193/*T. congolense*
Track 3: TREU 2177/*T. brucei*
Track 4: BAKIT 503 (group 2)
Track 5: BAKIT 427 (group 2)
Track 6: BAKIT 399 (group 2)
Track 7: BAKIT 500 (group 2)
Track 8: BAKIT 409 (group 1.6)
Track 9: BAKIT 461 (group 1.6)
Track12: BAKIT 423 (group 1.5)
Track13: BAKIT 467 (group 1.5)
Track14: BAKIT 382 (group 1.4)
Track15: BAKIT 401 (group 1.4)
Track16: BAKIT 508 (group 1.2)
Track17: BAKIT 100 (group 1.3)
Track18: BAKIT 505 (group 1.1)
Track19: BAKIT 134 (group 1.1)

Table 4.15. Hybridisation patterns of the *T. brucei* tubulin probe in *T. congolense* (2193), *T. brucei* (2177) and 14 *T. evansi* stocks.

Stock No.	2193	2177	503	427	399	500	409	461	423	467	382	401	508	100	505	134
Karyotype Group			**2**	**2**	**2**	**2**	**1.6**	**1.6**	**1.5**	**1.5**	**1.4**	**1.4**	**1.2**	**1.3**	**1.1**	**1.1**
Sample well	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Compression zone		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2.2 Mb	+	+				+			+	+	+	+		+	+	+
1.6 Mb			+	+	+		+	+			+	+	+		+	+
β tubulin pattern	**I**	**II**	**III**	**III**	**III**	**II**	**III**	**III**	**II**	**II**	**IV**	**IV**	**III**	**II**	**IV**	**IV**

Hybridisation patterns shown by the *T. brucei* rRNA coding region (λ104) gene probe are shown in **Figure 4.28**. Hybridisation was detected in Lambda DNA ladder and *S. cerevisiae* size standard markers. The λ 104 gene probe hybridised with the *T. congolense* DNA remaining in the sample well, 2.2 Mb, 1.6 Mb, 800 kb and 620 kb (Track 2, **Figure 4.28**). Hybridisation of the gene probe was detected in *T. brucei* DNA that remained in the sample well, ~2.4 Mb and 1.7 Mb (Track 3, **Figure 4.28**). In *T. evansi* the λ 104 gene probe hybridised with the DNA that stayed in the slot, the megabase size chromosomal DNA (~2.2 Mb, 1.6 Mb, 1.2 Mb) and some of the intermediate size chromosomal DNA between 670-950 kb size range. In all stocks used in the present study, hybridisation was not observed in the DNA sizes that stayed in the compression zone nor in the minichromosomes. It was observed that the hybridisation patterns shown by the λ104 gene probe varied among *T. evansi* stocks. The λ104 hybridisation patterns shown by *T. evansi* stocks were also different from those shown by *T. brucei* and *T. congolense*.

The *Trypanosoma brucei* aldolase gene probe hybridised with DNA trapped in the sample well in most of the trypanosome stocks used. Two *T. evansi* stocks, BAKIT 382 and 100 (Tracks 14 and 17 respectively, **Figure 4.29**) did not show any hybridisation with the Aldolase gene probe.

The Phospholipase C and Cysteine Proteinase gene probes did not hybridise with the trypanosome chromosomes separated by TAFE. **Figure 4.30** showed that there were no signs of hybridisation on chromosomal bands with phospholipase C in *T. evansi*, *T. brucei* and *T. congolense* stocks used in the study. The hybridisation reactions for the PLC gene probe were carried out on 4 different gels with high and low stringency washes, variable exposure times to the x-ray films and increased amounts of the gene probe was used.

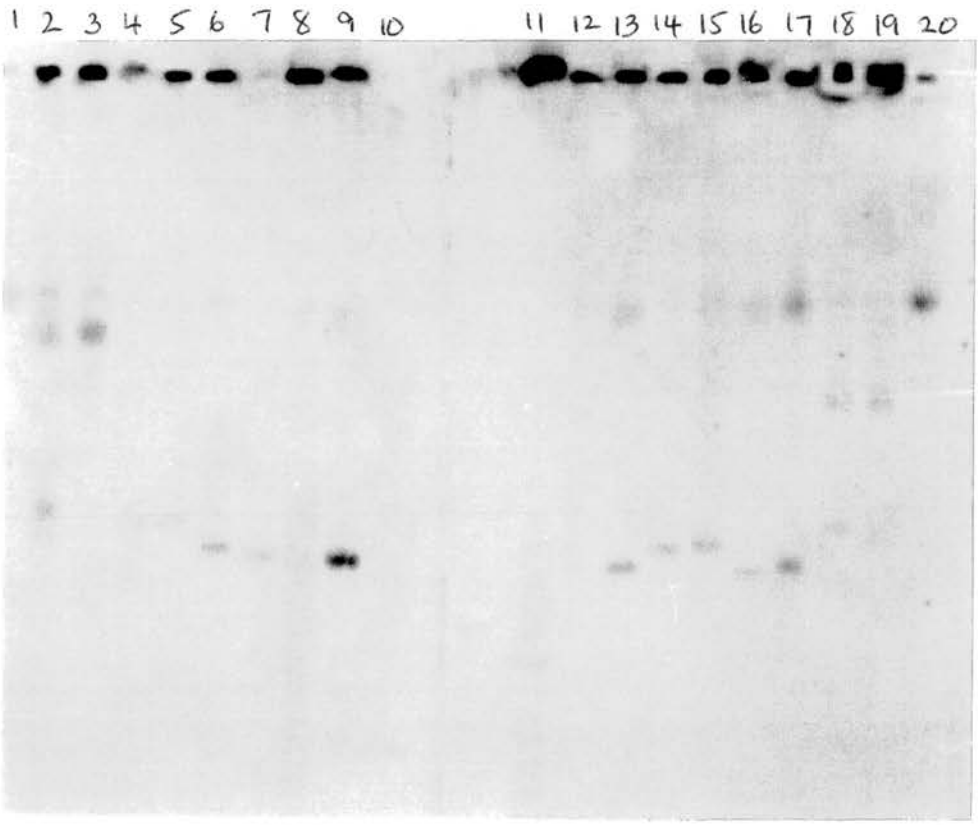


Figure 4.28. Southern blot hybridisation of *T. evansi*, *T. brucei* and *T. congolense* stocks separated by Transverse Alternating Field Electrophoresis with *T. brucei* rRNA coding region (λ104) gene probe.

Tracks 1, 10, 20: *Saccharomyces cerevisiae* size standard marker;
Track11: lambda DNA concatemer size standard
Track 2: TREU 2193/*T. congolense*
Track 3: TREU 2177/*T. brucei*
Track 4: BAKIT 503 (group 2)
Track 5: BAKIT 427 (group 2)
Track 6: BAKIT 399 (group 2)
Track 7: BAKIT 500 (group 2)
Track 8: BAKIT 409 (group 1.6)
Track 9: BAKIT 461 (group 1.6)
Track12: BAKIT 423 (group 1.5)
Track13: BAKIT 467 (group 1.5)
Track14: BAKIT 382 (group 1.4)
Track15: BAKIT 401 (group 1.4)
Track16: BAKIT 508 (group 1.2)
Track17: BAKIT 100 (group 1.3)
Track18: BAKIT 505 (group 1.1)
Track19: BAKIT 134 (group 1.1)

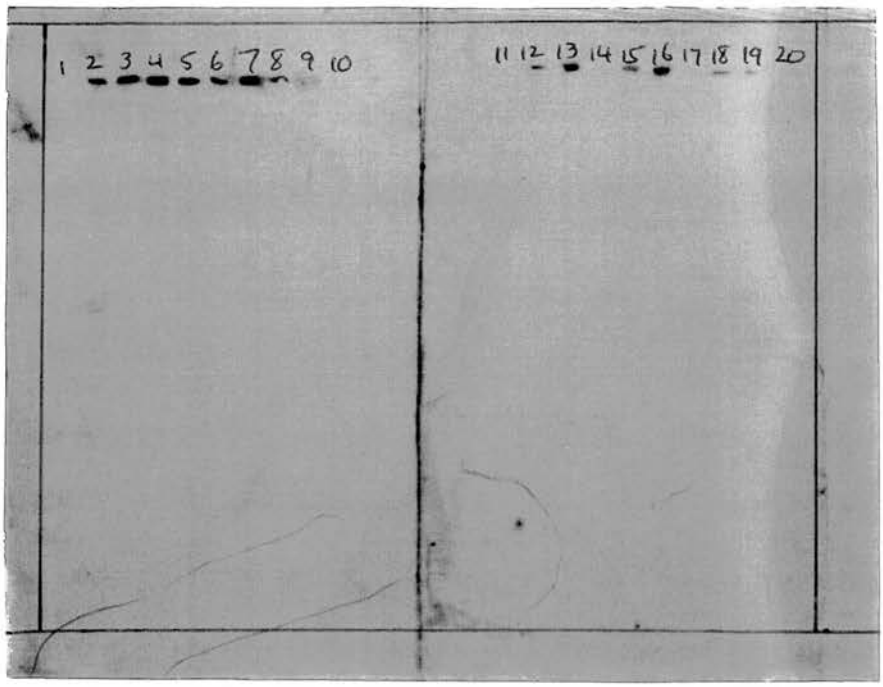


Figure 4.29. Southern blot hybridisation of *T. evansi*, *T. brucei* and *T. congolense* stocks separated by Transverse Alternating Field Electrophoresis with Aldolase gene probe.

Tracks 1, 10, 20: *Saccharomyces cerevisiae* size standard marker;
Track11: lambda DNA concatemer size standard
Track 2: TREU 2193/*T. congolense*
Track 3: TREU 2177/*T. brucei*
Track 4: BAKIT 503 (group 2)
Track 5: BAKIT 427 (group 2)
Track 6: BAKIT 399 (group 2)
Track 7: BAKIT 500 (group 2)
Track 8: BAKIT 409 (group 1.6)
Track 9: BAKIT 461 (group 1.6)
Track12: BAKIT 423 (group 1.5)
Track13: BAKIT 467 (group 1.5)
Track14: BAKIT 382 (group 1.4)
Track15: BAKIT 401 (group 1.4)
Track16: BAKIT 508 (group 1.2)
Track17: BAKIT 100 (group 1.3)
Track18: BAKIT 505 (group 1.1)
Track19: BAKIT 134 (group 1.1)

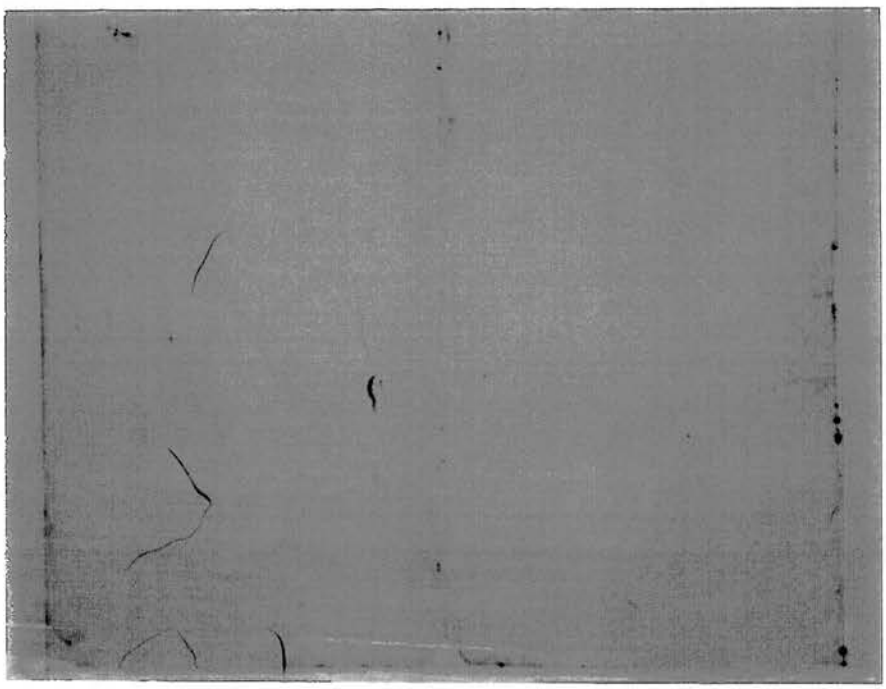


Figure 4.30. Southern blot hybridisation of *T. evansi*, *T. brucei* and *T. congolense* stocks separated by Transverse Alternating Field Electrophoresis with Cysteine Protease gene probe.

4.8.4. DISCUSSION

This study showed that the hybridisation patterns of PGI, λ104 and β tubulin gene probes in *T. evansi* differed from those of *T. brucei* and *T. congolense.* The glucose-6-phosphate isomerase (PGI) gene probe, which was prepared by labelling an amplification product of a DNA fragment (~450 bp) sequenced by Marchand *et al.* (1988) showed similar hybridisation patterns in the *T. brucei* and *T. evansi* stocks tested. The hybridisation signal of the PGI probe in *T. evansi*, however, was stronger in the intermediate size chromosomes than in the megabase size chromosomes. *Trypanosoma congolense* showed different hybridisation patterns of the PGI gene probe from those of *T. evansi* and *T. brucei*, the hybridisation signals in *T. congolense* was detected in the DNA remaining in the slot and the minichromosomal DNA. This had shown the closer relationships between the two species of the subgenus *Trypanozoon, T. brucei* and *T. evansi* than to *T. congolense* because the probe was derived from *T. brucei* PGI gene sequence. The hybridisation of the PGI gene probe to several bands indicated either that there are multiple PGI genes scattered on several chromosomes in *T. evansi* or *T. brucei* or there are many PGI-related sequences detected in different chromosome size. Gibson and Garside (1991) reported that the PGI gene probe (Marchand *et al.*, 1988) hybridised with the DNA that remained in the gel slot and chromosomes of megabase area in *T. evansi* stocks from Kenya, similarly in *T. brucei* (Marchand *et al.*, 1988; Gottesdiener *et al.*, 1990; Mensa-Wilmot, Hereld and Englund, 1990; Gibson and Garside, 1991; Gibson *et al.*, 1995). The presence of PGI genes in the intermediate size chromosomes in *T. evansi* and *T. brucei*, is in contrast to the results described by Gibson and Borst (1986) who did not find housekeeping genes in any chromosomes smaller than 700 kb. The different hybridisation signals between this study and other workers might be due to the differences in PFGE system used for fractionating the chromosomal DNA, this study used the TAFE system which results in finer separation than PFG or CHEF employed by other workers. It may also due to the gene probe used in this study was a labelled PCR product of a fragment of 450 bp of PGI gene sequence described by Marchand *et al.* (1988) whilst other workers used a clone of the whole sequence of the PGI gene as their probe.

The gene locations for the PLC and CP were not detected under any stringency washes performed in this study. Low stringency washes only produced high background. All of the post-hybridisational washes in this study were performed at high stringency (low salt concentration, high wash temperature) to obtain great specificity and remove the background. Reasons for non-detection of chromosomal gene localisation for probes, PLC and CP were unclear. It might be the non-radioactive label used in this study did not give as strong signal as the radioactive label used by other workers (Hide, 1995, personal communications).

The PLC gene probe in *T. brucei* chromosomes separated using the CHEF system, was detected in the chromosome size of ~2 Mb (Mensa-Wilmot *et al.*, 1990; Turner *et al.*, 1997) and in the ~1.1 Mb and ~1.5 Mb (Turner *et al.*, 1997); the hybridisation, however, was not detected in DNA remaining in the slot (Mensa-Wilmot *et al.*, 1990). It was further suggested that the PLC gene was only present in bloodstream form and not in procyclic form (Mensa-Wilmot *et al.*, 1990). The cysteine proteinase (CP) gene probe was located at *T. brucei* chromosomal DNA with the sizes range of ~2.2 Mb to ~3 Mb (Turner *et al.*, 1997).

The aldolase gene probe was located in the DNA that remained in the gel slot in *T. evansi*, *T. brucei* and *T. congolense* stocks used in the study. This was in agreement with the results reported in *T. brucei* (Gibson and Borst, 1986; Marchand *et al.*, 1988; Gottesdiener *et al.*, 1990; Gibson and Garside, 1991). However, in *T. cruzi* the ALD gene was located at the chromosome bands between 800-1,000 kb size range (Gibson and Miles, 1986).

The gene location of the *T. brucei* tubulin (β tubulin) in *T. evansi* was different from *T. brucei* and *T. congolense* used in this study. Results from the present study have shown a polymorphism in the hybridisation patterns with β tubulin gene probe. Variation in the β tubulin hybridisation patterns correlated with the 7 assigned karyotype groups of *T. evansi* stocks used. None of the hybridisation patterns detected in the 14 *T. evansi* stocks studied matched with that of *T. congolense*. Four *T. evansi* stocks (BAKIT 500, 423, 467 and 100) have identical β tubulin hybridisation patterns to that of *T. brucei*. The results indicated that *T. evansi* was more closely related to *T. brucei* than to *T. congolense*. Results from the present study were in agreement to those reported in *T. brucei* (Gibson and Garside, 1991; Gibson and Whittington, 1993; Gibson and Bailey, 1994; Gibson *et al.*, 1995) and *T. cruzi* (Engman *et al.*, 1987) who detected the β tubulin gene probe in the megabase size chromosomes.

The gene location of λ104 in *T. evansi* was in different chromosome size than that of *T. brucei*. The intermediate size chromosomes of *T. evansi* carry the λ104 gene whereas they were absent in the intermediate size chromosomes of *T. brucei*. Pairs of homologous chromosomes had been detected in *T. evansi*, based on the hybridisation of the λ104 gene marker (see tracks 4-7, 9 and 13-17 in **Figure 4.28**), two stocks BAKIT 505 and 134 (Tracks 18 and 19 respectively, **Figure 4.28**) showed three chromosomal bands hybridised with λ104. The λ104, which represents the ribosomal coding region (Hide *et al.*, 1990) is one of the repetitive DNA probes. The probe had been used to determine the relatedness of pairs of individuals and the epidemiological relationships between *T. brucei* stocks from Zambia and Kenya/Uganda, and detected subspecies differences between *T.b. gambiense* and *T.b. brucei* (Hide *et al.*, 1991).

Hybridisation with the λ104 and β tubulin gene probes had indicated the presence of homologous chromosomes in *T. evansi*. This study has also shown the polymorphism in the hybridisation patterns using genetic markers which correlated with karyotype groupings. The chromosomal localisation of the genetic markers used in the study therefore had provided more information on the chromosomal organisation in *T. evansi*.

CHAPTER FIVE

CHARACTERISATION OF *TRYPANOSOMA EVANSI* STOCKS BY RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS

5.1. INTRODUCTION

Genomic fingerprinting by arbitrarily primed polymerase chain reaction (AP-PCR, Welsh and McClelland, 1990) or random amplified polymorphic DNA (RAPD, Williams *et al.*, 1990) analysis can provide a rapid screening method for the detection of DNA polymorphisms between different genomes. The PCR based technique uses single short oligonucleotides of arbitrary sequence as primers. After amplification by RAPD-PCR, products were electrophoresed on agarose gels and the products detected by ethidium bromide. A major advantage of RAPD is that no prior knowledge of the organism DNA sequence to be studied is required. Compared to the DNA sequencing, the RAPD analysis can identify many markers, the effort and the costs involved are modest (Tibayrenc *et al.*, 1993).

The RAPD technique was first applied to generate genomic fingerprints to identify species and strain differences in bacteria (*Staphylococcus* and *Streptococcus*) and varieties of rice, *Oryza sativa*, (Welsh and McClelland, 1990). Application of RAPD technology was also reported by Williams *et al.* (1990) who constructed genetic maps of variety of species (human, plants and bacteria) based on the polymorphisms generated by RAPD markers. The polymorphisms in the RAPD profiles generated specific patterns and allowed detection of the differentiation between closely related strains within the same species (Welsh and McClelland, 1990). It was suggested (Welsh and McClelland, 1990, Williams *et al.*, 1990; Hadrys *et al.*, 1992) that the RAPD method can be applied for the determination of breeding programme, genetic mapping, studies of the population genetics and epidemiology.

The RAPD analysis was applied to detect polymorphisms among stocks in *T. brucei* (Mathieu-Daude, Stevens, Welsh *et al.*, 1995), *T. vivax* (Dirie, Murphy and Gardiner, 1993b), *T. rangeli* (Steindel *et al.*, 1994), Anuran trypanosomes isolated from American toads (Lun and Desser, 1996a), *Leishmania braziliensis* (Gomes, Macedo, Pena *et al.*, 1995) and

Lucilia cuprina, an ectoparasite of sheep (Stevens and Wall, 1997). Similarities in the RAPD patterns were shown among stocks of parasites, which correlated with the isolation locality from which they were isolated. On the contrary, studies carried out by Carrasco, Frame, Valente *et al.* (1996) detected homogeneity in the RAPD patterns among *T. cruzi* stocks from widely separated areas in Central and South America. It was suggested, however, that RAPD analysis has limited application on epidemiological studies because of the lack of repeatability of the overall fingerprint due to the competition of many priming sites in the genome for a single primer (Bishop *et al.*, 1993).

The RAPD analysis was also used for taxonomic phylogenetic studies (Mathieu-Daude *et al.*, 1995) in *T. brucei* subspecies. The *T. brucei* subspeciation detected by RAPD analysis (Mathieu-Daude *et al.*, 1995; Waitumbi and Murphy, 1993; Kanmogne *et al.*, 1996) matched with isoenzyme pattern analysis (Tait *et al.*, 1985; Stevens and Godfrey, 1992) and hybridisation with *T. brucei* antigen gene probes (Paindavoinne *et al.*, 1986b; 1989) and with *T. brucei* repetitive DNA probes (Hide *et al.*, 1990; 1991). The RAPD pattern of *T.b. brucei* was similar to that of *T.b. rhodesiense* and differed from that shown by *T.b. gambiense* stocks studied.

Three geographical types of *T. congolense*, Savannah, Kilifi and West African type have been confirmed by RAPD analysis (Waitumbi and Murphy, 1993), which had been shown by Gibson *et al.* (1988) on the hybridisation with *T. congolense* repetitive DNA probes. However, Waitumbi and Murphy (1993) did not detect polymorphisms among *T. evansi* stocks by RAPD analysis.

Oury, Dutrait, Bastrenta *et al.* (1997) designed internal primers generated from a common fragment in *T. cruzi* stocks amplified by RAPD to be used as probes to target specific taxonomic levels, such as clone, family of related clone or species and for diagnosis. The specific RAPD pattern was also suggested to be applied in the species identification of *Theileria parva* (Bishop *et al.*, 1993), *Babesia bovis* (Lew, *et al.*, 1997) and differential diagnosis of *T. rangeli* from *T. cruzi* (Steindel *et al.*, 1994). It was suggested that the RAPD markers provided a quick method for generating genetic maps and population analysis (Michelmore, Paran and Kesseli, 1991) because the RAPD analysis detects more variation than restriction fragment length polymorphism (RFLP) (Kanmogne *et al.*, 1996) and by multi-locus enzyme electrophoresis (MLEE) (Mathieu-Daude *et al.*, 1995; Stevens and Tibayrenc, 1995).

The main advantage of using RAPD analysis, other than there is no prior knowledge of DNA sequence needed, is the use of a universal set of primers that can be applied to amplify for a wide variety of species (Williams *et al.*, 1990). However, it is important to optimise the RAPD conditions. Many factors had been recognised to affect the RAPD banding patterns

including Mg^{++} concentration, primer concentration and the number of cycles in the amplification reaction.

This chapter describes, firstly, the use of an RAPD analysis with the primer ILO525 (5'-CGGACGTCGC) which was used by Waitumbi and Murphy (1993) to determine inter- and intra-species differences in *T. brucei* and *T. evansi* originating from Kenya. In the present study, the identical sequence of the primer was synthesized and used to amplify *T. evansi* stocks isolated in Indonesia in order to examine differences among the Indonesian stocks tested. Other *T. evansi* stocks from Kenya, Brazil and Sudan and a *T. brucei* stock were also included in the study.

The second part of this chapter describes studies on RAPD analysis using a set of commercially available 10-mer arbitrary primer obtained from Genosys. The RAPD standardisation was carried out to determine the optimal concentration of Mg^{++} in the buffer and the concentration of primer used for the amplification. Other conditions were performed according to the RAPD procedures described by Waitumbi and Murphy (1993). Each of the primers was used to amplify a set of 9 *T. evansi* stocks which were representative of 9 different karyotypes. The aim of this study is to find suitable primer(s) capable of detecting polymorphism among *T. evansi* stocks in the RAPD analysis.

The third part of this chapter presents results on the application of a selected primer, with potential to detect inter-stock differences, to test the stability of the RAPD profiles in *T. evansi* relapse populations and to examine the RAPD patterns in the stocks collected from widely distributed areas in Indonesia. The *Trypanosoma evansi* DNA samples used in the study were obtained from agarose blocks used in the pulsed-field gel electrophoresis (PFGE). To assess the application of RAPD analysis on samples without prior employment of the long steps for DNA purification, trypanosome samples were also prepared directly from the cryopreserved trypanosome stabilates. The purpose of the study was to maximise the use of RAPD analysis for epidemiological studies on a greater number of stocks from widely distributed areas, which were stored in liquid nitrogen.

5.2. OPTIMISATION OF RAPD ANALYSIS USING PRIMER ILO 525

5.2.1. STANDARDISATION OF RAPD ANALYSIS

The RAPD analysis was standardised to determine the optimal Mg^{2+} concentration in the standard PCR buffer (Waitumbi and Murphy, 1993) and the number of reaction cycles.

5.2.1.1. MATERIALS AND METHODS

5.2.1.1.1. *Trypanosoma*

Agarose embedded DNA prepared from *T. evansi* (BAKIT 431) was used in the study. Approximately 10 ng of DNA contained in 1/8 block was used in all RAPD amplification reaction. The estimation of the DNA content in agarose blocks was based on the assumption that the genome size of *T. evansi* was approximately 40 Mb (= 4 x 10^7 bp) containing approximately 0.04 pg per trypanosome. All of the agarose blocks were prepared from 2 x 10^6 trypanosomes containing approximately 80 ng DNA, with 10 ng per 1/8 of agarose block used as template DNA in the RAPD reaction.

5.2.1.1.2. Primer

A single oligonucleotide, ILO525 (5'CGGACGTCGC3') (Waitumbi and Murphy, 1993), was synthesised by Cruachem (UK) and used as primer. The primer was used at 0.6 μM per reaction.

5.2.1.1.3. Magnesium chloride concentration

Six different $MgCl_2$ concentrations (1, 1.5, 2, 2.5, 3 and 4 mM) were incorporated into the PCR buffer (10 mM Tris pH 8.3, 50 mM KCl, 0.05% NP40, 0.05% Tween20). Other components in each of the RAPD reaction include: 200 μM dNTPs, 0.6 μM primer (ILO525), 2.5 units *Taq* DNA polymerase (Boehringer Mannheim, Germany) and approximately 10 ng DNA contained in 1/8 agarose block. The RAPD reaction was carried out for 30 cycles as described by Waitumbi and Murphy (1993).

Each RAPD amplification reaction was undertaken in a final volume of 50 μl reaction. The reaction was carried out in sterile conditions; the PCR pipette tips (Finntip, Lab Sciences, UK) and 0.5 ml microcentrifugation tubes were UV sterilised using Gene Linker (BioRad, UK). In each amplification reaction, sterile 18.2 MΩ water was added first into the tube in the desired volume; then the 10x PCR buffer, primer and dNTPs solutions were added at 5 μl each (**Table 5.1**). The *Taq* DNA polymerase (Boehringer Mannheim, Germany), isolated and purified from *Thermus aquaticus*, was added at 2.5 units (5 μl) into each reaction tube. Approximately 75 μl of mineral oil was added on top of each of the reaction mixtures and finally the *T. evansi* DNA was added last. The reaction mixtures were then centrifuged

(Hawksley, UK) for 3 seconds at 13,000 rpm before placing them in the thermal cycler (OmniGene Thermal Cycler, Hybaid).

The thermal cycler was programmed for 30 cycles; at 94^0 C for 1 minute for denaturation; 40^0 C for 1 minute and 30 seconds for annealing and 72^0 C for 2 minutes for extension (Waitumbi and Murphy, 1993). The amplification products were stored in a refrigerator (10^0 C) until required for further analysis and incubated at 50^0 C in a waterbath for 5 minutes to melt the LMP agarose.

5.2.1.1.4. Effect of high number of reaction cycles

Three different PCR buffers with each containing three different $MgCl_2$ concentrations (1.5, 3 and 4 mM) were used in each amplification reaction. Other components including 200 μM dNTPs; 0.6 μM of ILO525 primer; 2.5 units of Taq DNA polymerase (Boehringer Mannheim, Germany) and approximately 10 ng of *T. evansi* (BAKIT 431) DNA contained in 1/8 agarose block (~2.5 μl) were added into the reaction. Each amplification reaction with a final volume of 50 μl was carried out under sterile conditions. The amplification was carried out using two reaction cycles: 30 and 45, each used following conditions: 94^0 C for 1 minute for denaturation; 40^0 C for 1 minute for annealing and 72^0 C for 2 minutes for extension.

Table 5.1. Standard RAPD reaction composition using ILO525 primer.

Composition	Volume added (μl)	Final concentration in PCR reaction
18.2 MΩ H_2O	32	-
RAPD buffer 10 x	5	1 x
dNTPs (2 mM stock)	5	200 μM
Primer	5	0.6 μM
Taq (Boehringer Mannheim)	0.5	2.5 units
DNA (1/8 block)	2.5	~10 ng
Total Volume	50	

5.2.1.1.5. RAPD detection

The RAPD products were detected by 5% polyacrylamide gel electrophoresis. The gel was composed of 2.5 ml of 30% acrylamide bis, 2 ml of 5x Loening buffer stock, 5.5 ml of 18.2 MΩ water, 10 μl of TEMED and 70 μl of 10% ammonium persulfate. The 1 x Loening buffer

is 35.8 mM Tris, 39.2 mM Sodium dihydrogen phosphate and 5 mM EDTA disodium dihydrate.

Each amplification product was used without purification at a volume of 5 μl and put into a 0.5 ml microfuge tube containing 1 μl of 6x gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol). The amplification product-gel loading buffer mixture was loaded into the polyacrylamide gel well at a volume of 3 μl and the 1 kb standard marker (Gibco, BRL) was loaded at a concentration of 1 μg/mm lane width. The electrophoresis was carried out in the presence of 1 x Loening buffer, in a Mini Protean II (BioRad, UK) at 200 volts for 15 minutes or until the bromophenol blue reaches the bottom of the gel.

The gel was visualised by silver staining (Herring, Inglis, Ojeh, *et al.*, 1982; Sanguinetti, Neto and Simpson, 1994). After electrophoresis, the polyacrylamide gel was fixed for 10 minutes in 10% (v/v) alcohol and 0.5% (v/v) acetic acid glacial, and then followed by staining in 0.19% (w/v) silver nitrate for 10 minutes. After staining, the gel was rinsed twice with distilled water then the colour was developed (for 5 to 10 minutes) by soaking the gel in the buffer containing 3% (w/v) sodium hydroxide and 0.75% (v/v) formaldehyde. All incubation steps in silver staining procedures were carried out with gentle shaking at room temperature. The colour development was stopped after 5-10 minutes by replacing the developing buffer with stopping buffer containing 0.75% (w/v) anhydrous sodium carbonate for 3 to 5 minutes.

The gel was then transferred into a sealable plastic bag, for easy manipulation, then the banding patterns were photographed using a Polaroid MP-4 Land Camera and filter no 45 (blue filter). The f-stop was set at 4.5 with 3-4 seconds exposure time and 45 seconds developing time. The estimation of the band sizes was carried out by comparing against the 1 kb standard size marker (Gibco, BRL) and determined using BioImage® whole band analyser software (Millipore, USA).

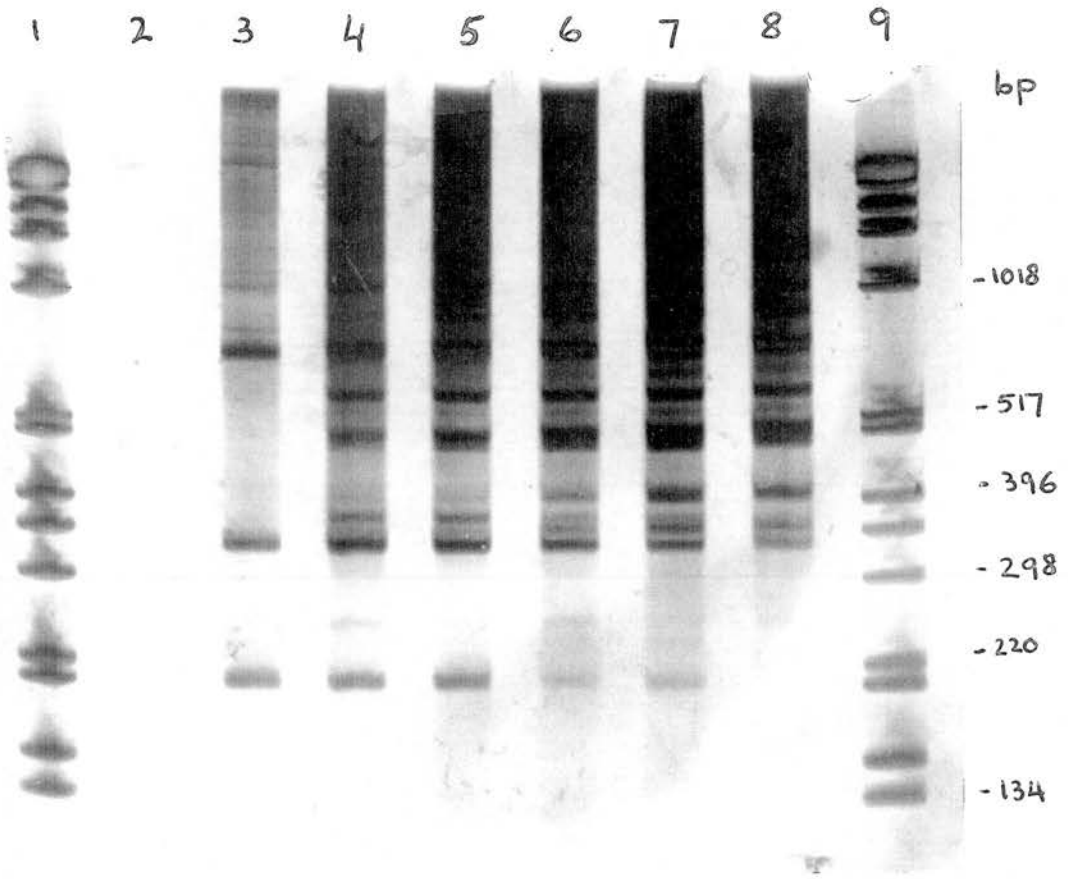
5.2.1.2. RESULTS

5.2.1.2.1. Effect of Mg^{++} concentration

The effects of changes in the Mg^{++} concentration in the PCR reaction carried out for 30 cycles were presented in **Figure 5.1**. Significantly fewer bands were seen in the reaction containing the lowest (1 mM) $MgCl_2$ concentration, and the band at ~200 bp showed as a very intensely stained band. However, the band gradually decreased its intensity with the increasing concentration of Mg^{++} in the reaction, while the number of detected bands increased in the RAPD products containing higher Mg^{++} concentrations from 1 mM to 2.5 mM. The banding patterns of the amplification products containing 2.5, 3 and 4 mM $MgCl_2$ were similar.

Figure 5.1. The effects of changes in the Mg^{++} concentration in the Random Amplified Polymorphic DNA (RAPD) analysis using a single arbitrary primer ILO525 (5'CGGACGTCGC3').

Tracks 1, 9: 1 kb standard DNA size marker
Track 2: Control tube (no DNA)
Track 3: RAPD amplification using 1 mM $MgCl_2$
Track 4: RAPD amplification using 1.5 mM $MgCl_2$
Track 5: RAPD amplification using 2 mM $MgCl_2$
Track 6: RAPD amplification using 2.5 mM $MgCl_2$
Track 7: RAPD amplification using 3 mM $MgCl_2$
Track 8: RAPD amplification using 4 mM $MgCl_2$


1
2
3
4
5
6
7
8
9
bp
1018
517
396
298
220
134

The bands above 740 bp were not clearly separated in any of the amplification products. The bands above 300 bp in the 3 mM $MgCl_2$ reaction were more intensely stained compared to those of the reactions containing lower concentrations of $MgCl_2$. The banding pattern in the reaction containing 4 mM $MgCl_2$ showed faint bands below 300 bp compared to those containing 1.5 to 3 mM $MgCl_2$. In all $MgCl_2$ conditions, the bands above 740 bp were not well resolved. The reaction containing 3 mM $MgCl_2$ was chosen for further analysis because of the clear banding pattern shown by the reaction. The band sizes shown by *T. evansi* DNA amplified by the RAPD analysis containing various Mg^{++} concentrations is summarised in **Table 5.2**.

Table 5.2. The effect of $MgCl_2$ concentration in RAPD amplification reaction generated by ILO525 primer.

Band No.	Band Size (bp)	$MgCl_2$ (mM)					
		1.0	1.5	2.0	2.5	3.0	4.0
1	740	+*	±	+	+	+	+
2	640	-	-	±	+	+	+
3	615	-	+*	+*	+*	+*	+*
4	540	-	-	±	±	+	+
5	500	-	+*	+*	+*	+*	+*
6	490	-	+	+*	+*	+*	+*
7	390	-	±	+	+*	+*	+
8	350	-	+	+	+	+	±
9	340	-	-	±	+	+*	+
10	350	-	+*	+*	+	+	±
11	320	+*	+*	+*	+*	+*	+
12	300	-	±	±	±	±	±
13	270	-	-	-	-	+	±
14	250	-	+	+	±	±	±
15	230	-	±	±	±	±	±
16	200	+*	+*	+*	±	+	±

Note: - Band absent
+ Band present
± Faint band present
+* Intensely stained band present

5.2.1.2.2. Effect of high number of cycles

In all conditions (variable $MgCl_2$ concentrations and the reaction cycle numbers) the bands above 740 bp were not well resolved. The smallest DNA fragment (~200 bp) observed in the amplification product was most intensely stained in the reaction containing lowest Mg^{++} concentration, which gradually decreased its intensity with the increasing Mg^{++} concentration and increasing the number of detectable bands.

Differences in the banding patterns and the number of bands were not observed in the reactions run for either 30 cycles or 45 cycles when an equal concentration of Mg^{++} was used. For example, similar banding patterns were observed between the RAPD products containing 1.5 mM $MgCl_2$ that was amplified for 30 cycles and that amplified for 45 cycles. A blank (no DNA) control, however, showed a banding pattern that differed from those of the amplification products containing *T. evansi* DNA.

This study also indicated that increasing the number of cycles in the RAPD reaction from 30 to 45 cycles did not affect the banding patterns under this RAPD analysis condition and similar banding patterns were obtained in the RAPD products containing either 3 mM or 4 mM Mg^{++}. It appeared that 3 mM $MgCl_2$ in the PCR buffer was the optimal concentration for RAPD analysis. Overall, the study had suggested the use of 3 mM Mg^{++} concentration and 30 PCR cycles to be optimal for the present study.

5.2.1.3. DISCUSSION

The study had shown the greatest effect of the RAPD amplification reaction was caused by varying the Mg^{++} concentration. The Mg^{++} affects primer annealing, enzyme activity and fidelity. Although the 2 mM $MgCl_2$ contained in the reaction yield an equal number of bands to that of the 3 mM $MgCl_2$ concentration (**Figure 5.1**), the reaction containing 3 mM $MgCl_2$ improved some bands intensity that are faint in the lower $MgCl_2$ concentration without "losing" the already present band (200 bp) in 2 mM $MgCl_2$ reaction. The Mg^{++} binds proportionally to dNTPs, which is necessary for DNA polymerase reaction. Eckert and Kunckel (1993) suggested that the highest fidelity of DNA polymerase is achieved by using equal concentrations of $MgCl_2$ and dNTPs. However, it was suggested (Innis and Gelfand, 1990) that PCR amplification should contain 0.5 to 2.5 mM Mg^{++} over the total dNTP concentration.

The optimum number of cycles in PCR is related to the starting amount of DNA and the efficiency of each amplification step (Innis and Gelfand, 1990; Taylor, 1993). Too many cycles in PCR increases the amount of background products and too few cycles causes low

yield product. Tyler *et al.* (1997) reported that increasing the number of cycles to 45 in RAPD analysis in bacterial pathogen resulted in increasing the number of background bands as the intensities of all bands were also increased. It had been reported that the use of different numbers of cycles, or cycle time, and the use of different thermocycler brands, resulted in different amplification products (MacPherson *et al.*, 1993). This study, however, showed stability in the number of bands in both products run for 30 and 45 cycles containing the same primer concentration. The product run for 45 cycles showed a more intense banding pattern than that of the product run for 30 cycles. It appeared that different primer concentrations had greater influence in RAPD analysis than the number of reaction cycles. The band stability, which was not observed in bacterial pathogen, might be due to the larger genome size in trypanosomes as had been suggested by Tyler *et al.* (1997).

5.2.2. ANALYSIS OF *T. EVANSI* AND *T. BRUCEI* STOCKS USING ILO525 PRIMER

This study was carried out to determine the RAPD patterns polymorphisms in *T. evansi* stocks from Indonesia, Sudan, Kenya and Brazil using the ILO525 primer that had been used by Waitumbi and Murphy (1993). This study also determined inter-species differences in the RAPD patterns between *T. evansi* and *T. brucei*.

5.2.2.1. MATERIALS AND METHODS

5.2.2.1.1. *Trypanosoma*

Agarose embedded DNA prepared from nine *T. evansi* stocks from Indonesia, one stock each from Kenya, Brazil, Sudan and a *T. brucei* stock were selected for the RAPD amplification (**Table 5.3**).

Table 5.3. *Trypanosoma brucei* and *T. evansi* stocks used in RAPD analysis.

Stock no.	Trypanosome species	Country	Isolation locality	Host species
BAKIT 381	*T. evansi*	Indonesia	North Sumatra	Buffalo
BAKIT 424	*T. evansi*	Indonesia	North Sumatra	Buffalo
BAKIT 431	*T. evansi*	Indonesia	Lampung	Cattle
BAKIT 374	*T. evansi*	Indonesia	Central Java	Buffalo
BAKIT 385	*T. evansi*	Indonesia	North Sumatra	Buffalo
BAKIT 402	*T. evansi*	Indonesia	North Sumatra	Buffalo
BAKIT 517	*T. evansi*	Indonesia	Madura	Buffalo
BAKIT 417	*T. evansi*	Indonesia	Lampung	Cattle
BAKIT 399	*T. evansi*	Indonesia	North Sumatra	Buffalo
TREU 1810	*T. evansi*	Kenya		Camel
BAKIT 229	*T. evansi*	Sudan		Camel
TREU 2187	*T. evansi*	Brazil		Dog
TREU 2177	*T. brucei*	Uganda		Cattle

5.2.2.1.2. RAPD amplification and detection

Each RAPD reaction was carried out at a final volume of 50 μl. The standard PCR buffer containing 3 mM of $MgCl_2$ was used in the amplification reaction. A 'master mix' was prepared for 13 reactions. Each reaction contains sterile deionised water (18.2 MΩ), 10x PCR buffer, ILO525 primer (0.6 μM), dNTPs (200 μM) and 2.5 units of *Taq* DNA polymerase (Boehringer Mannheim). The trypanosome DNA samples were added into each tube after the addition of mineral oil. The RAPD amplification were carried out at 94^0 C for 1 minute for denaturation; 40^0 C for 1 minute and 30 seconds for annealing and 72^0 C for 2 minutes for extension for 30 cycles (Waitumbi and Murphy, 1993).

The banding patterns of the amplification products generated by the primer ILO525 were visualised in silver stain of 5% polyacrylamide gel and photographed. The band sizes were determined and the banding patterns were compared as has been described previously in Section 5.2.1.1.

5.2.2.2. RESULTS

5.2.2.2.1. *T. evansi* Indonesian stocks

Two RAPD patterns were detected in the 9 *T. evansi* stocks isolated from Indonesia and amplified by RAPD using primer ILO525. A total of 22 and 23 bands within the size range of 180 bp to 1020 bp were observed in the silver stained polyacrylamide gel loaded with the amplification product (**Figure 5.2**). The pattern is characterised by the presence of 10 intensely stained bands at approximately 620 bp, 605 bp, 537 bp; 504 bp, 489 bp; 395 bp; 360 bp; 342 bp; 324 bp and 196 bp. Variable patterns were detected in bands between 196-324 bp. Stocks showing pattern 1 consisted of *T. evansi* stocks BAKIT 402, 381 and 399, all isolated in North Sumatra. Pattern 2 stocks consisted of BAKIT 517/Madura, 417/Lampung, 431/Lampung, 424/North Sumatra, 385/North Sumatra and 374/Central Java. The difference between the two patterns was the presence of a double band at 274 and 260 bp in pattern 2.

5.2.2.2.2. Comparison with other *T. evansi* and *T. brucei* stocks

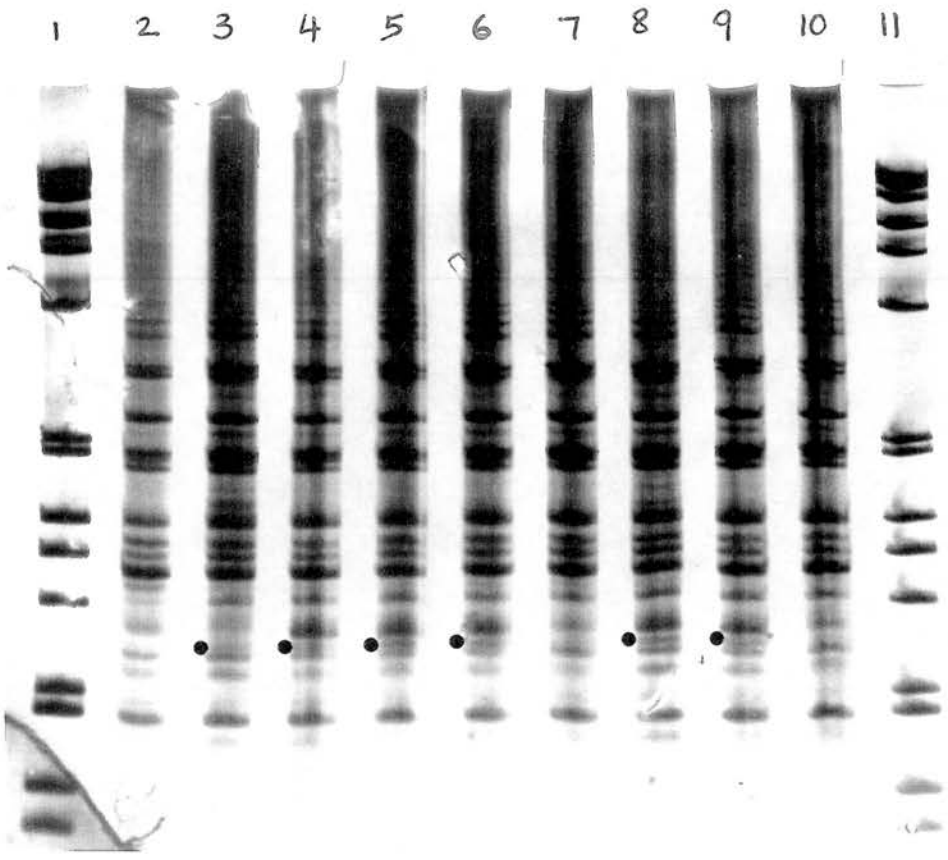
The *T. evansi* stocks from Indonesia (4) were compared with stocks from Kenya (1), Brazil (1), Sudan (1) and a *T. brucei* stock from Uganda (**Figure 5.3**). Polymorphisms in the RAPD patterns were observed among *T. evansi* stocks from Indonesia, Kenya, Brazil and Sudan. The summary of the comparison of band sizes revealed by the amplification products generated by the primer ILO525 is presented in **Table 5.4**.

The RAPD banding patterns in *T. brucei* and *T. evansi* stocks were similar on the bands above 600 bp. *Trypanosoma evansi* stocks differed from *T. brucei* by the presence of additional bands at approximately 537 bp, 489 bp and 301 bp. The banding patterns in all *T. evansi* stocks, however, were similar in the bands above 320 bp. The *T. brucei* stock amplified with this primer produce a characteristic intense band at 290 bp. This band was not present in the *T. evansi* stocks studied.

The *T. evansi* stocks from Indonesia amplified with the primer ILO525 showed two different patterns. The first pattern detected in 3 out of 4 stocks (BAKIT 381, 424 and 399), showed similar banding pattern to the *T. evansi* stock from Kenya. The second pattern detected in one *T. evansi* stock (BAKIT 431) from Indonesia, differed from other trypanosome stocks tested by the presence of an intensely stained band at 280 bp. The *T. evansi* stock from Brazil differed from the other stocks tested by the presence of a thick and intensely stained band at 260 bp and the Sudanese *T. evansi* differed from the rest of the *T. evansi* and *T. brucei* stocks by the presence of 234 bp.

Figure 5.2. The random Amplification Polymorphic DNA patterns of *Trypanosoma evansi* stocks from Indonesia amplified by ILO525 primer.

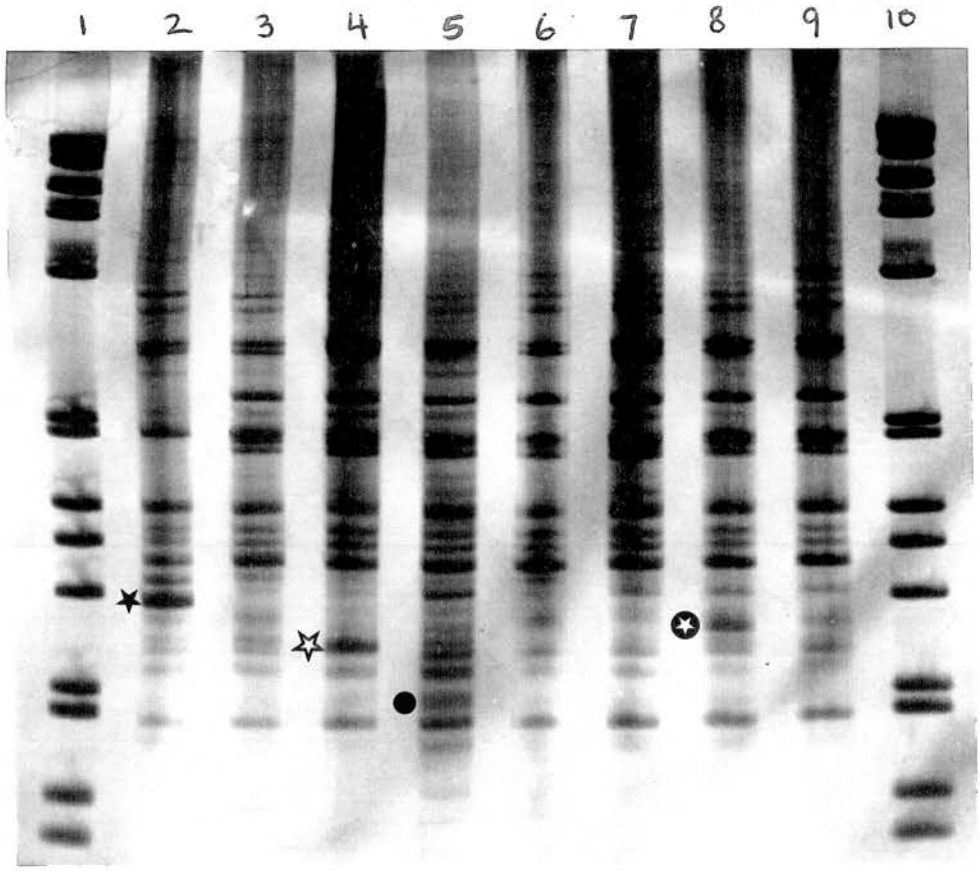
Tracks 1, 11: 1 kb standard DNA size marker
Track 2: BAKIT 381
Track 3: BAKIT 424
Track 4: BAKIT 431
Track 5: BAKIT 374
Track 6: BAKIT 385
Track 7: BAKIT 402
Track 8: BAKIT 517
Track 9: BAKIT 417
Track10: BAKIT 399



• 274 bp

Figure 5.3. The random Amplification Polymorphic DNA patterns of *Trypanosoma evansi* and *Trypanosoma brucei* stocks amplified by ILO525 primer.

Tracks 1, 10: 1 kb standard DNA size marker
Track 2: TREU 2177 (*T. brucei*)
Track 3: TREU 1810 (*T. evansi*, Kenya)
Track 4: TREU 2187 (*T. evansi*, Brazil)
Track 5: BAKIT 229 (*T. evansi*, Sudan)
Track 6: BAKIT 381
Track 7: BAKIT 424
Track 8: BAKIT 431
Track 9: BAKIT 399



★ 291 bp
☆ 260 bp
✪ 280 bp
● 234 bp

Table 5.4. Comparison of the banding patterns shown by the amplification products generated by the primer ILO525 of *T. evansi* from Kenya, Brazil, Sudan and Indonesia with that of *T. brucei* from Uganda.

Band No.	Band Size (bp)	*T. brucei*	*T. evansi*				
			Kenya	Brazil	Sudan	Indonesia	
						1	2
1	1020	+	+	+	+	+	+
2	940	+	+	+	+	+	+
3	823	+	+	+	+	+	+
5	734	+	+	+	+	+	+
6	620	++	++	++	++	++	++
7	605	++	++	++	++	++	++
8	560	+	±	±	+	±	+
9	537	-	++	++	++	++	++
10	520	+	+	+	+	±	+
11	504	++	+++	+++	+++	+++	+++
12	489	-	++	++	++	++	++
13	460	-	-	-	+	-	-
14	420	-	-	-	+	-	-
15	395	++	+++	+++	+++	+++	+++
16	360	+	++	++	++	+	++
17	342	+	++	++	++	+	++
18	324	++	+++	+++	+++	+++	+++
19	310	++	++	±	±	+	±
20	301	-	+	±	++	+	+
21	291	++++	-	-	-	-	-
22	280	±	+	±	+	+	++
23	274	±	+	-	+	±	+
25	260	±	+	+++	+	+	+
26	254	±	+	++	++	+	+
27	234	-	-	±	++	-	-
28	196	+	+	++	++	++	++
29	180	±	-	-	++	±	±

Note: - Band absent
Band intensity ranging from ± (faint) to ++++ (very intense).

5.2.2.3. DISCUSSION

The study had shown the intra-species differences in the RAPD patterns revealed by the amplification with the single arbitrary primer ILO525. Polymorphism in the RAPD patterns were observed in *T. evansi* stocks originating from Kenya, Sudan, Brazil and Indonesia. The study had suggested that polymorphism in the RAPD patterns among the *T. evansi* studied could be correlated with the location from where the stocks were isolated. The RAPD patterns generated by the primer ILO525 showed differences among *T. evansi* stocks isolated in Indonesia. Results from this study were contrary to those obtained by Waitumbi and Murphy (1993) who did not detect polymorphisms in the RAPD patterns among *T. evansi* stocks from Kenya. The differences in the RAPD patterns among *T. evansi* stocks from Indonesia might be due to the geographical isolation of the stocks collected. Both studies, however, agreed that the primer ILO525 generated different amplification products between *T. evansi* and *T. brucei.*

The present study showed that the RAPD pattern of the *T. brucei* stock differed from that of *T. evansi* by the presence of an intense band at 290 bp in *T. brucei* and the absence of bands in 537 bp, 489 bp and 301 bp. Waitumbi and Murphy (1993), however, found a less intense band of 287 bp in *T. evansi* and a characteristic 273 bp intense band in *T. brucei.* The presence of a characteristic band at 273 bp in *T. brucei* found by Waitumbi and Murphy (1993) was not detected in this study. The different results obtained by both studies might be caused by several factors that influence the fidelity in RAPD analysis, as has been pointed out by Tyler *et al.* (1997), including Mg^{++} concentrations, primer concentrations, enzyme source and concentration, DNA extraction methods, DNA concentration, the model of thermocycler used and inter laboratories conditions. The RAPD reaction condition (Mg^{++} concentration, DNA concentration and preparation, primer concentration) in the present study was similar to that applied by Waitumbi and Murphy (1993). Two conditions in the RAPD analysis carried out in the present study, however, were different from those of Waitumbi and Murphy: principally the DNA polymerase source and the model of thermocycler. The present study used the BioImage® whole band analyser computer software to determine and compare the RAPD banding patterns among the stocks analysed, which might be different from the detection method carried out by Waitumbi and Murphy (1993). This study had shown the importance of applying identical conditions when carrying out the analysis in different laboratories.

5.3. OPTIMISATION OF RAPD ANALYSIS OF *T. EVANSI* USING A SET OF 10-MER ARBITRARY PRIMER

Further study to determine intra-species differences in the RAPD patterns was carried out using a set of 10-mer primers synthesized by Genosys. The effect of two different primer concentrations and various Mg^{++} concentrations in the PCR standard buffer was investigated.

5.3.1. STANDARDISATION OF RAPD ANALYSIS

5.3.1.1. MATERIALS AND METHODS

5.3.1.1.1. *Trypanosoma evansi* DNA

The DNA template for the RAPD reactions was obtained from agarose embedded DNA of a *T. evansi* stock (BAKIT 431) from North Sumatra. Approximately 10 ng DNA contained in 1/8 agarose block was used in each RAPD reaction.

5.3.1.1.2. Effect of primer concentration

Two different primer concentrations were tested in the RAPD analysis: 0.1 and 0.3 μM. One of the primer sets was chosen (5'-GAGACGTCCC) for the optimisation of the primer concentration. Other PCR reaction components as shown in **Table 5.5**, consist of PCR buffer containing 3 mM $MgCl_2$; 200 μM dNTPs and 2 units of DNA polymerase, isolated and purified from *Thermus brokianus* by DynaZyme (Finland).

Table 5.5. Standard RAPD reaction composition using Genosys primers

Composition	Volume added (μl)	Final concentration
H_2O	31.5	-
RAPD buffer 10x	5	1x
dNTPs (2 mM stock)	5	200 μM
Primer	5	0.3 μM
DynaZyme	1	2 units
DNA (1/8 block)	2.5	~ 10 ng
Total volume	50	

Approximately 10 ng of *T. evansi* DNA contained in 1/8 agarose block (~2.5 μl) was used. A control tube (no DNA) was included in the amplification reaction containing 0.3 μM of primer. The RAPD amplification was carried out at 94^0 C for 1 minute for denaturation, 40^0 C for 1 minute for annealing and 72^0 C for extension for 30 cycles. The amplification products were electrophoresed, without purification, in a 5% polyacrylamide gel and the 1 kb standard (Gibco, BRL) was included in the electrophoresis as the size marker. The gel was visualised by silver staining and photographed as described previously in Section 5.2.1.1.5. The band sizes were determined and the banding patterns were compared using BioImage® Whole Band Analyser computer software.

5.3.1.1.3. Effect of Mg^{++} concentration

Various concentrations of $MgCl_2$ (1, 1.5, 2, and 3 mM) were added into the PCR buffer to determine its concentration for the RAPD analysis. The RAPD-PCR buffer was prepared according to the standard PCR buffer described by Waitumbi and Murphy (1993) with substitution of various $MgCl_2$ concentrations added to the buffer. Other components in this reaction include: 200 μM dNTPs; 0.3 μM of primer (5'-GAGACGTCCC); 2 units of DNA polymerase (DynaZyme, Finland) and approximately 10 ng of *T. evansi* DNA contained in 1/8 agarose block (~2.5 μl) (**Table 5.5**). The RAPD amplification was carried out at 94^0 C for 1 minute for denaturation; 40^0 C for 1 minute and 30 seconds for annealing and 72^0 C for extension for 30 cycles (Section 5.2.1.1). The amplification products were electrophoresed in the 5% polyacrylamide gel with the inclusion of the 1 kb standard (Gibco, BRL) as the DNA size marker and visualised by silver staining described in Section 5.2.1.1. The band sizes were determined and the banding patterns were compared using BioImage® Whole Band Analyser computer software.

5.3.1.1.4. Stability of the RAPD pattern

To investigate the stability in the RAPD products, agarose DNA samples from relapse populations of an uncloned *T. evansi* infection, as described in Chapter 4 (Section 4.6) were amplified with the primer 5'GAGACGTCCC. Each 50 μl of amplification reaction was composed of the 10x standard PCR buffer containing 3 mM $MgCl_2$; 200 μM dNTPs; 0.3 μM of primer (5'GAGACGTCCC); 2 units of DNA polymerase (DynaZyme, Finland) and approximately 10 ng of *T. evansi* DNA contained in the 1/8 agarose block. The RAPD amplification was carried out at 94^0 C for 1 minute for denaturation, 40^0 C for 1 minute for annealing and 72^0 C for 2 minutes for extension for 30 cycles. The RAPD products were separated in the 5% polyacrylamide gel electrophoresis and visualised by silver staining. The 1 kb standard DNA marker (Gibco, BRL) was used as the DNA size marker in the electrophoresis.

5.3.1.2. RESULTS

5.3.1.2.1. Effect of primer concentration

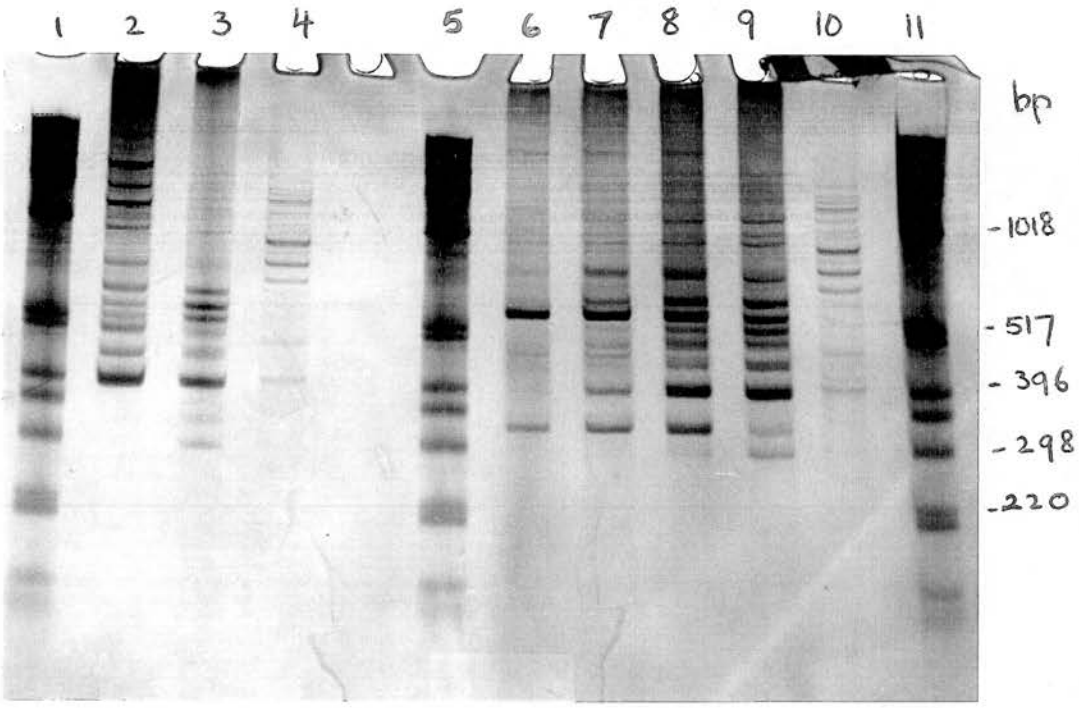
The effect of two different primer concentrations on the RAPD banding patterns of *T. evansi* is presented in **Figure 5.4**. The gel showed well separated bands of the *T. evansi* stock (BAKIT 431) amplified with primer GEN-046. The amplification product containing 0.1 μM primer (Track 2, **Figure 5.4**) showed a clear banding pattern of the bands above 370 bp. Thirteen bands were observed in the size range of 370 bp to ~ 2,000 bp; eight of the bands were located below 1,100 bp. The RAPD product amplified with 0.3 μM primer (Track 3, **Figure 5.4**) showed 12 well separated bands in the size range of 280-1,100 bp. Four additional bands in the region below 1,000 bp were detected at 620 bp, 440 bp, 310 bp and 280 bp were detected when the primer concentration was increased to 0.3 μM. The band sizes of the amplified DNA in **Figure 5.4** (Tracks 2 and 3) were summarised in **Table 5.6**. The amplification product with no DNA (Track 4, **Figure 5.4**) showed some bands, but the banding pattern was different from the products containing *T. evansi* DNA.

5.3.1.2.2. Effect of Mg^{++} concentration

Overall, it was shown that increasing the $MgCl_2$ concentration from 1 mM to 3 mM in the buffer resulted in the presence of several additional bands (**Figure 5.4**). The amplification product containing 1 mM $MgCl_2$ (Track 6, **Figure 5.4**) showed an unclear banding pattern; only two (310 bp and 540 bp) of them were strong bands. The banding pattern was clearer when the $MgCl_2$ concentration was increased to 1.5 mM (Track 7, **Figure 5.4**) with 10 bands being detected in the size range of 300-1,100 bp. The amplification product generated by the RAPD reaction containing 2 mM $MgCl_2$ (Track 8, **Figure 5.4**) showed clear banding pattern in the bands between 300-1,000 bp, with an addition of a faint band at 280 bp which was not present in the RAPD products generated by the reactions containing 1 and 1.5 mM $MgCl_2$. The amplification products containing 3 mM $MgCl_2$ (Track 9, **Figure 5.4**) showed a similar banding pattern to that of the 2 mM $MgCl_2$ reaction. The band at 280 bp was more clearly detected in the product containing 3 mM $MgCl_2$ than that of 2 mM $MgCl_2$. The RAPD product containing 3 mM $MgCl_2$ showed best separated bands with 12 bands being observed in the size range of 280-1100 bp compared to those of reactions containing lower $MgCl_2$ concentrations. **Table 5.6** summarised the band sizes in the amplification products containing a variety of $MgCl_2$ concentrations in the RAPD reaction (Tracks 6-9 in **Figure 5.4**). The amplification product in the reaction that did not contain DNA (Track 10, **Figure 5.4**) showed bands with a different pattern to those products containing *T. evansi* DNA.

Figure 5.4. The effects of two different primer concentrations and Mg^{++} concentrations on Random Amplified Polymorphic DNA banding patterns of *T. evansi* stocks amplified with GEN 046 primer (5'-GAGACGTCCC).

Tracks 1, 5, 11: 1 kb standard size marker
Track 2: RAPD amplification using 0.1 μM primer
Track 3: RAPD amplification using 0.3 μM primer
Track 4: No DNA, 0.3 μM primer
Track 6: RAPD amplification using 1 mM $MgCl_2$ and 0.3 μM primer
Track 7: RAPD amplification using 1.5 mM $MgCl_2$ and 0.3 μM primer
Track 8: RAPD amplification using 2 mM $MgCl_2$ and 0.3 μM primer
Track 9: RAPD amplification using 3 mM $MgCl_2$ and 0.3 μM primer
Track 10: No DNA, RAPD amplification using 3 mM $MgCl_2$ and 0.3 μM primer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10
11
bp
1018
517
396
298
220

Table 5.6. Effect of primer and Mg^{++} concentrations on RAPD banding patterns of *T. evansi* (BAKIT 431) DNA amplified by a single arbitrary primer 5' GAGACGTCCC.

Band size (bp)	Primer		$MgCl_2$ concentrations (mM)			
	0.1 μM	0.3 μM	1	1.5	2	3
1100	+	±	±	±	+	+
850	+	±	±	±	+	+
620	-	±	±	+*	+	+
570	+	+	-	+*	+*	+*
540	+	+	+*	+*	+*	+*
510	+	+	-	±	+	+
480	+	+	-	-	+	+
440	-	±	±	+	+	+
420	+	+	-	+	+	+
374	+*	+*	-	+*	+*	+*
310	-	+	+*	+*	+*	+
280	-	+	-	-	±	+

Note: - : Band absent
± : Faint band
+ : Band present
+* : Intensely stained band

5.3.1.2.3. RAPD pattern stability

RAPD pattern polymorphisms was not observed with the *T. evansi* populations collected from relapse populations. Identical banding patterns were observed with the presence of 13 bands at 1100 ; 850 ; 620 ; 570 ; 540 ; 510 ; 500 ; 480 ; 440 ; 420 ; 370 ; 310 and 280 bp.

5.3.1.3. DISCUSSION

5.3.1.3.1. Effect of increasing primer and magnesium concentration

The present study showed that increasing the primer concentration from 0.1 μM to 0.3 μM also increases the number of amplified bands smaller than 400 bp and reduces the number of larger bands (above 500 bp). One of the parameters that influence the reproducibility of RAPD analysis is the primer concentration. This study had shown that lower primer concentration (0.1 μM) had resulted in a banding pattern of bands of higher molecular size in the 5% polyacrylamide gel than that of the higher (0.3 μM) primer concentration. This might

be due to the presence of the non-specific background DNA fragments detected as high molecular size bands of above 1,000 bp. Welsh and McClelland (1990) proposed that in RAPD analysis the use of a single arbitrary primer and low temperature allow primers to anneal at many different sites with a variety of mismatches. In this study, the primer might have annealed most efficiently with higher molecular size DNA and the sequences between these positions were amplified. Only the sites that efficiently primed were amplified and predominated in the RAPD cycles. Because of insufficient primer concentration in the reaction the annealing of the primer was less effective. Meanwhile the DNA polymerase and dNTPs continued their activity. This may result in amplifying the non-primed DNA fragment detected as high molecular size bands, because the amplification products can act as primers for subsequent cycles in RAPD amplification. It was suggested (Bell and DeMarini, 1991) that the background bands detected in RAPD products were the random-length, high-molecular weight fragments resulting from extension and random termination of annealing events after most of the oligonucleotide primer had been converted to PCR product.

The effect of different $MgCl_2$ concentrations in PCR has been described above in the RAPD analysis using ILO525.

5.3.1.3.2. RAPD pattern stability

Stability in the RAPD profiles was seen in the relapse populations, which showed identical banding patterns with the primary isolate. This shows that the RAPD profile is stable and the trypanosome stock can be collected at any stage of infection and subjected to RAPD for stock characterisation. This result, however, was different from the *T. evansi* karyotype study, where polymorphisms in karyotype patterns were detected in relapse populations of a single infection. This study has shown that the RAPD analysis did not detect changes in antigenic variation as shown by karyotype analysis and analysis of surface variation antigenic types. Stability of the RAPD pattern was also reported in *Candida albicans* stocks (Bostock *et al.*, 1993). The RAPD patterns in three *C. albicans* stocks, two of them were collected 2 days later, showed similar patterns. The *C. albicans* stocks tested showed different karyotype patterns when they were subjected to the PFGE. It was suggested that the differences shown by PFGE were probably due to the genetic rearrangements, or the two strains coexisted, which were indistinguishable by RAPD analysis (Bostock *et al.*, 1993). This had shown that the karyotype pattern detected more variations in *T. evansi* stocks than the RAPD analysis.

5.3.4. RAPD ANALYSIS OF *T. EVANSI* STOCKS USING GENOSYS 10-MER ARBITRARY PRIMERS

The study was carried out to determine the single primer(s) that can be used to detect polymorphisms in RAPD patterns among *T. evansi* stocks from Indonesia.

5.3.4.1. MATERIALS AND METHODS

5.3.4.1.1. *T. evansi* stocks

DNA from *T. evansi* stocks from Indonesia listed in **Table 5.3** were used in this study. Agarose embedded *T. evansi* DNA prepared for PFGE was amplified with each single arbitrary primer.

5.3.4.1.2. Primers

A set of 10-mer arbitrary oligonucleotides was obtained from Genosys and the sequence of each primer is shown in **Table 5.7**. The primers were used at a final concentration of 0.3 μM.

Table 5.7. Primer sequences of the Genosys 10-mer arbitrary primers used in the study.

Primer	Sequence
Gen 3-70/3629-042	5'-TCCCTGTGCC
Gen 3-70/3629-044	5'-GCTCTCCGTG
Gen 3-70/3629-046	5'-GAGACGTCCC
Gen 3-70/3629-048	5'-GTATGCCGCG
Gen 3-70/3629-050	5'-GCACCGAACG
Gen 3-70/3629-052	5'-CCGGCGTATC
Gen 3-70/3629-054	5'-AGCCTGACGC
Gen 3-70/3629-056	5'-GCTCTGGCAG
Gen 3-70/3629-058	5'-CGCACTCGTC
Gen 3-70/3629-060	5'-CTGTCCGGTC

5.3.4.1.3. RAPD amplification and detection

The composition of each amplification reaction in the RAPD analysis has been described in **Table 5.5**. The RAPD amplification procedure and detection of amplification products were carried out according to the standard procedures described in section 5.2.1.1.

5.3.4.1.4. RAPD pattern analysis

The estimation of band sizes of amplification products was carried out by comparison against the 1 kb standard marker preparation (Gibco, BRL) and analysed using BioImage® Whole Band Analyser computer software package (Millipore, USA) as described in Chapter 3, Section 3.3.

5.3.4.2. RESULTS

The results obtained from RAPD analysis of *T. evansi* stocks with the 10 Genosys primers (**Table 5.7**) showed that 8 out of the 10 primers used did not show any polymorphism in the banding patterns of the 9 *T. evansi* stocks when subjected to the RAPD analysis. The *T. evansi* DNA amplified by primer GEN-042 (**Figure 5.5**); -044 (**Figure 5.6**); -048 (**Figure 5.8**); -050 (**Figure 5.9**); -052 (**Figure 5.10**); -054 (**Figure 5.11**); -058 (**Figure 5.13**) and -060 (**Figure 5.14**) gave identical banding patterns for all *T. evansi* stocks analysed. Each primer generated different patterns when used for amplifying the same set of *T. evansi* stocks.

The amplification products generated by primer GEN–046 (**Figure 5.7**) showed 4 different RAPD patterns while GEN-056 (**Figure 5.12**) produced two different banding patterns. In many occasions, in all amplification products with all primers, bands larger than 1,000 bp were excluded in the analysis because of their inconsistent banding patterns in this region.

Figure 5.7 showed polymorphisms in the banding patterns of *T. evansi* amplified with the primer GEN-046. At least 12 bands in the size range of 240-1,100 bp were observed and the presence of the bands larger than approximately 1,000 bp were not consistent. The RAPD patterns shown by the amplification products with the primer GEN-046 were characterised by the presence of unstable bands at approximately 570 bp; 420 bp and 240 bp. Twelve stable bands (1100 , 850 , 620 , 540 , 510 , 500 , 480 , 440 , 374 , 330 , 310 , 280 bp) present in *T. evansi* stocks amplified with primer GEN-046 with a very intense band at 374 bp.

Four RAPD patterns were noted on the basis of the presence and absence of the unstable bands in the size range of 230-1,100 bp. Pattern A consists of the amplification product of *T.*

evansi stock BAKIT 399 (Track 10, **Figure 5.7**). This pattern has a total of 15 bands, which consisted of the 12 stable bands and three unstable bands at 570, 420 and 240 bp.

Pattern B consists of the amplification products of *T. evansi* stocks BAKIT 381 (Track 2, **Figure 5.7**) and 402 (Track 7, **Figure 5.7**). The RAPD pattern B has a total of 14 bands consisting of the 12 stable bands and two unstable bands at 570 bp and 240 bp bands; the band at 420 bp is absent.

The third pattern (pattern C) was shown by the RAPD products of BAKITs 424; 374; 385 and 417 (Tracks 3, 5, 6 and 9 respectively in **Figure 5.7**). RAPD pattern C is characterised by the presence the 12 stable bands and two unstable of bands at 570 bp and 420 bp; the band at 240 bp is absent. This pattern has a total of 14 bands in the size range between 240-1,100 bp.

Pattern D which has 13 bands was shown by the amplification product of *T. evansi* stocks BAKITs 431 and 517 (Tracks 4 and 8 respectively, **Figure 5.7**). RAPD pattern D is characterised by the presence of the 12 stable bands and one unstable band at 420 bp; two unstable bands at 570 bp and 230 bp are absent. The band sizes of the four RAPD patterns shown in **Figure 5.7** are presented in **Table 5.8**.

Table 5.8. Random amplified DNA patterns in *T. evansi* stocks generated by the primer GEN-046.

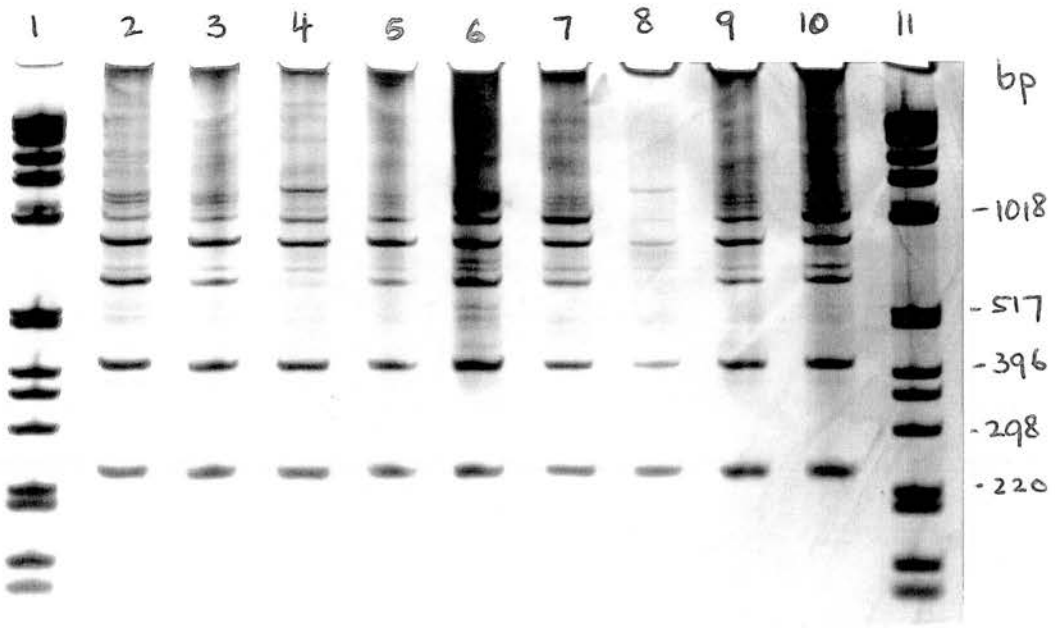
Band No.	Band Size (bp)	RAPD pattern			
		A	B	C	D
1	1,100	+	+	+	+
2	850	+	+	+	+
3	620	+	+	+	+
4	570	++	+	+	-
5	540	+	+	+	+
6	510	+	+	+	+
7	500	+	+	+	+
8	480	+	+	+	+
9	440	+	+	+	+
10	420	+	-	+	+
11	374	+++	+++	+++	+++
12	330	±	±	±	±
13	310	+	+	+	+
14	280	+	+	+	+
15	240	+	+	-	-

Note: Band intensities ranging from ± (faint) to +++ (intensely stained).
-: Bands absent

Figure 5.5. Random Amplified Polymorphic DNA patterns of *Trypanosoma evansi* stocks amplified by primer GEN-042 (5'-TCCCTGTGCC).

Tracks 1, 11: 1 kb size standard marker
Track 2: BAKIT 381
Track 3: BAKIT 424
Track 4: BAKIT431
Track 5: BAKIT 374
Track 6: BAKIT 385
Track 7: BAKIT 402
Track 8: BAKIT 517
Track 9: BAKIT 417
Track10: BAKIT 399

Figure 5.6. Random Amplified Polymorphic DNA patterns of *Trypanosoma evansi* stocks amplified by primer GEN-044 (5'-GCTCTCCGTG). Tracks 1-11: As in Figure 5.5.


1
2
3
4
5
6
7
8
9
10
11
bp
1018
517
396
298
220

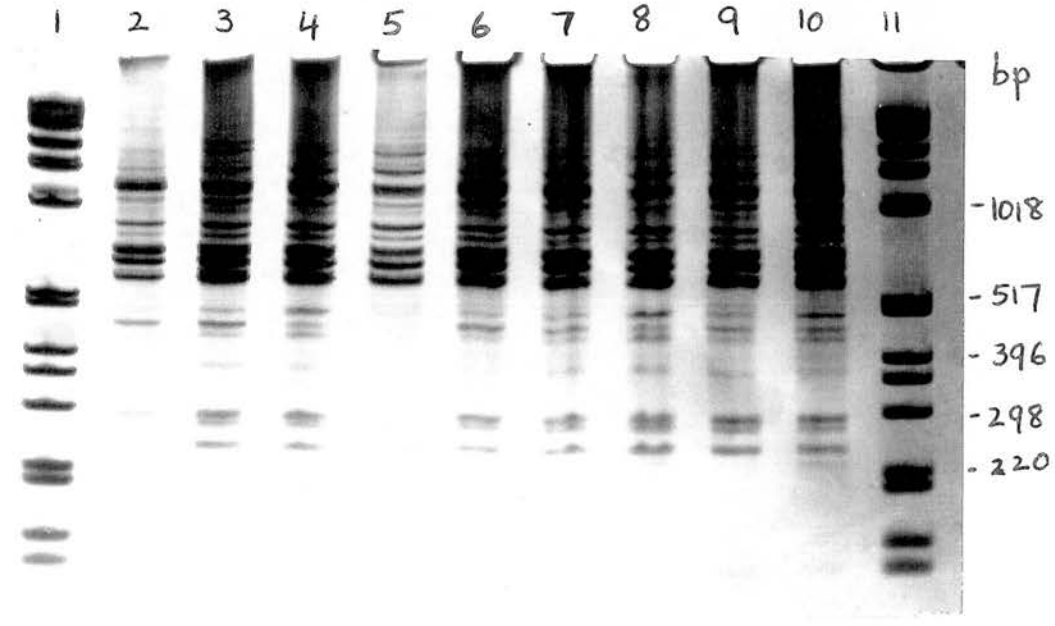
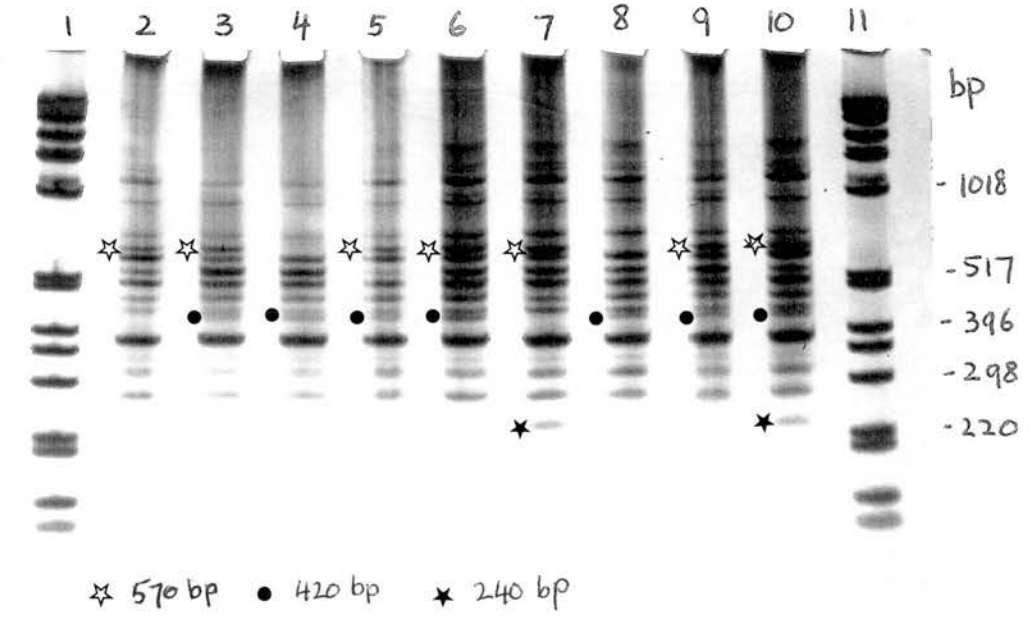
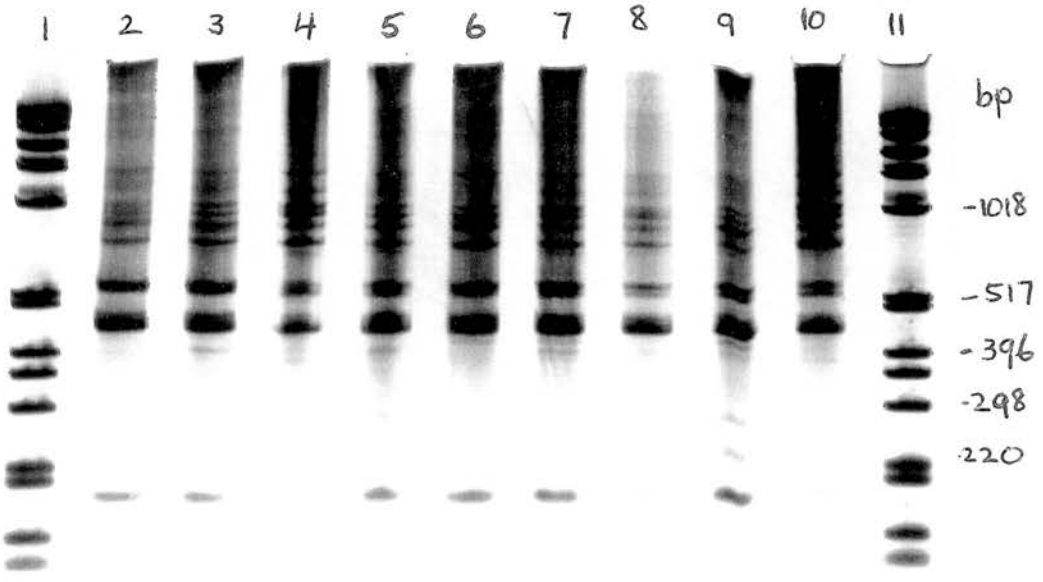

1
2
3
4
5
6
7
8
9
10
11
bp
1018
517
396
298
220

Figure 5.7. Random Amplified Polymorphic DNA patterns of *Trypanosoma evansi* stocks amplified by primer GEN-046 (5'-GAGACGTCCC).

Tracks 1, 11: 1 kb size standard marker
Track 2: BAKIT 381
Track 3: BAKIT 424
Track 4: BAKIT431
Track 5: BAKIT 374
Track 6: BAKIT 385
Track 7: BAKIT 402
Track 8: BAKIT 517
Track 9: BAKIT 417
Track10: BAKIT 399

Figure 5.8. Random Amplified Polymorphic DNA patterns of *Trypanosoma evansi* stocks amplified by primer GEN-048 (5'-GTATGCCGCG). Tracks 1-11: As in Figure 5.7.


1
2
3
4
5
6
7
8
9
10
11
bp
1018
517
396
298
220
570 bp
420 bp
240 bp


1
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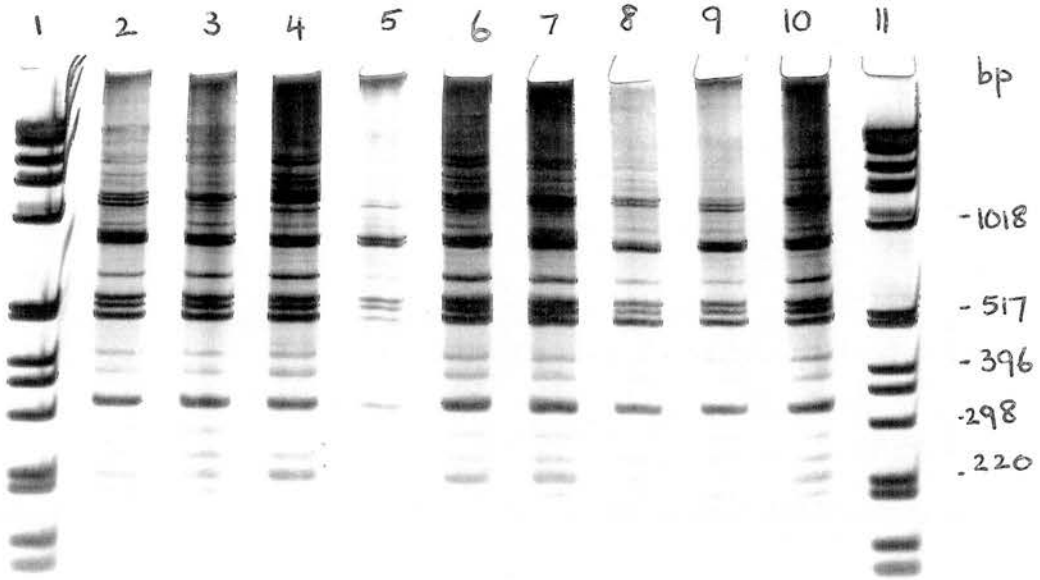
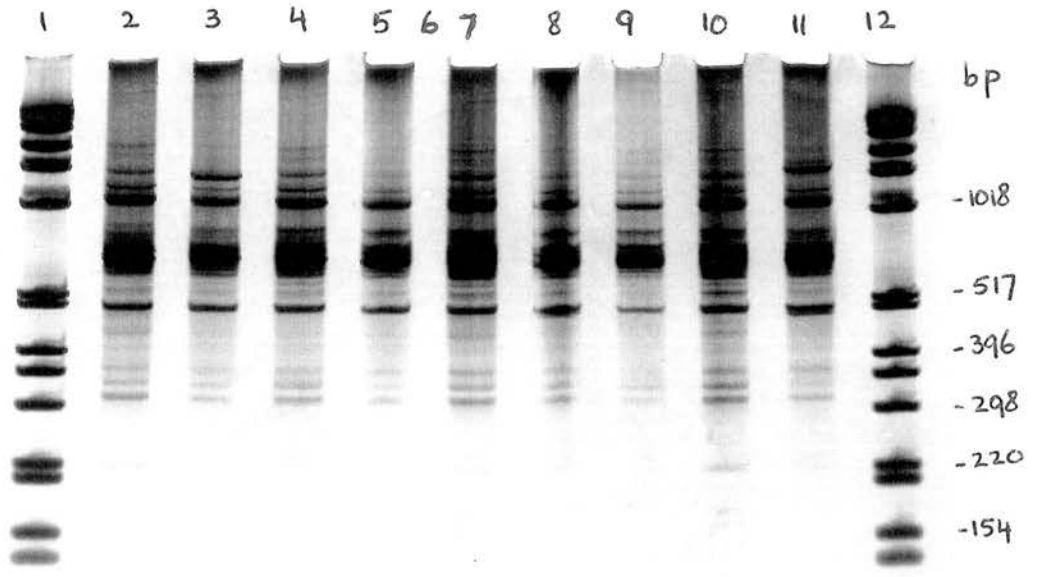
3
4
5
6
7
8
9
10
11
bp
1018
517
396
298
220

Figure 5.9. Random Amplified Polymorphic DNA patterns of *Trypanosoma evansi* stocks amplified by primer GEN-050 (5'-GCACCGAACG).

Tracks 1, 11: 1 kb size standard marker
Track 2: BAKIT 381
Track 3: BAKIT 424
Track 4: BAKIT431
Track 5: BAKIT 374
Track 6: BAKIT 385
Track 7: BAKIT 402
Track 8: BAKIT 517
Track 9: BAKIT 417
Track10: BAKIT 399

Figure 5.10. Random Amplified Polymorphic DNA patterns of *Trypanosoma evansi* stocks amplified by primer GEN-052 (5'-CCGGCGTATC). Tracks 1-11: As in Figure 5.9.


1
2
3
4
5
6
7
8
9
10
11
bp
1018
517
396
298
220


1
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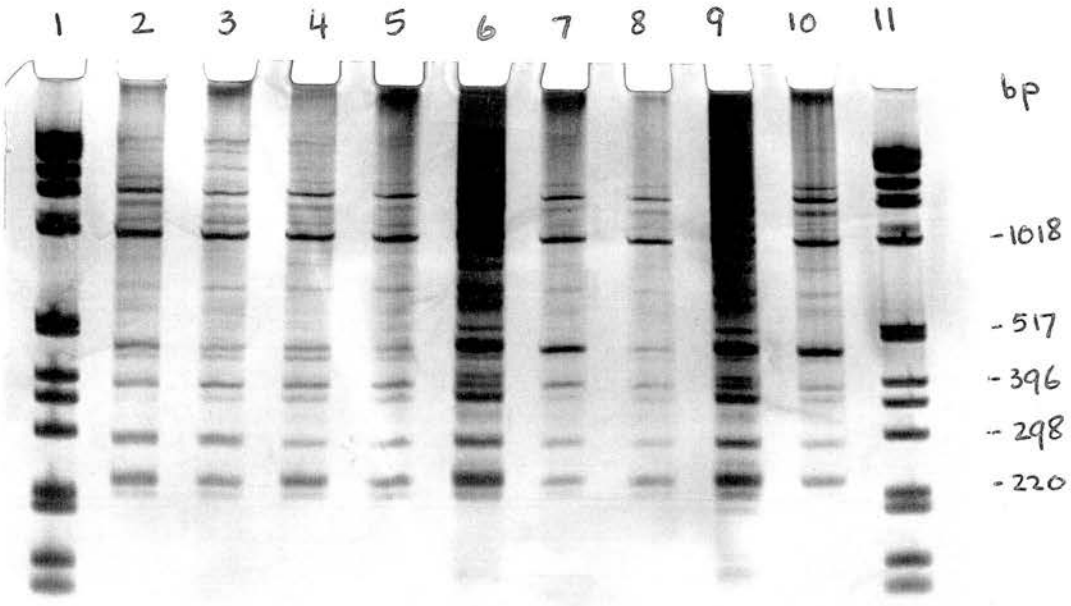
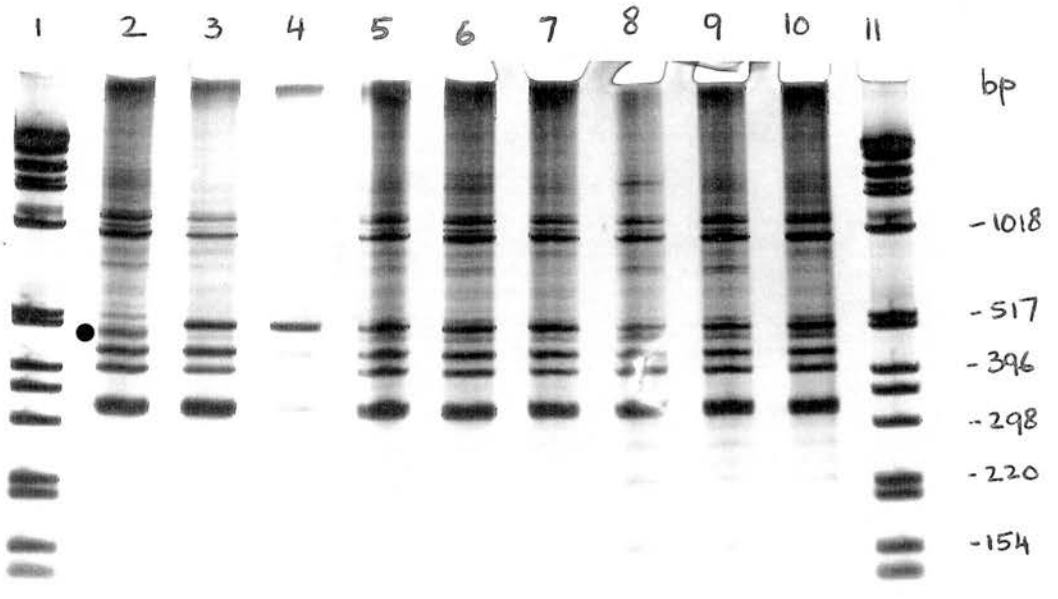
6
7
8
9
10
11
12
bp
1018
517
396
298
220
154

Figure 5.11. Random Amplified Polymorphic DNA patterns of *Trypanosoma evansi* stocks amplified by primer GEN -054 (5'-AGCCTGACGC).

Tracks 1, 11: 1 kb size standard marker
Track 2: BAKIT 381
Track 3: BAKIT 424
Track 4: BAKIT431
Track 5: BAKIT 374
Track 6: BAKIT 385
Track 7: BAKIT 402
Track 8: BAKIT 517
Track 9: BAKIT 417
Track10: BAKIT 399

Figure 5.12. Random Amplified Polymorphic DNA patterns of *Trypanosoma evansi* stocks amplified by primer GEN-056 (5'-GCTCTGGCAG). Tracks 1-11: As in Figure 5.11.


1 2 3 4 5 6 7 8 9 10 11
bp
1018
517
396
298
220

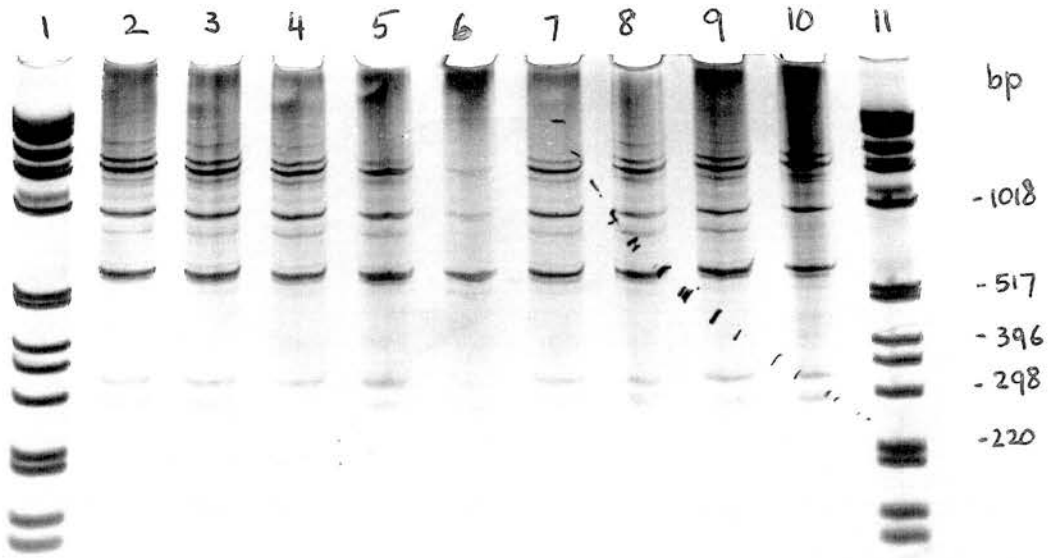

1 2 3 4 5 6 7 8 9 10 11
bp
1018
517
396
298
220
154

● 380 bp

Figure 5.13. Random Amplified Polymorphic DNA patterns of *Trypanosoma evansi* stocks amplified by primer GEN-058 (5'-CGCACTCGTC).

Tracks 1, 11: 1 kb size standard marker
Track 2: BAKIT 381
Track 3: BAKIT 424
Track 4: BAKIT431
Track 5: BAKIT 374
Track 6: BAKIT 385
Track 7: BAKIT 402
Track 8: BAKIT 517
Track 9: BAKIT 417
Track10: BAKIT 399

Figure 5.14. Random Amplified Polymorphic DNA patterns of *Trypanosoma evansi* stocks amplified by primer GEN-060 (5'-CTGTCCGGTC). Tracks 1-11: As in Figure 5.13.


1
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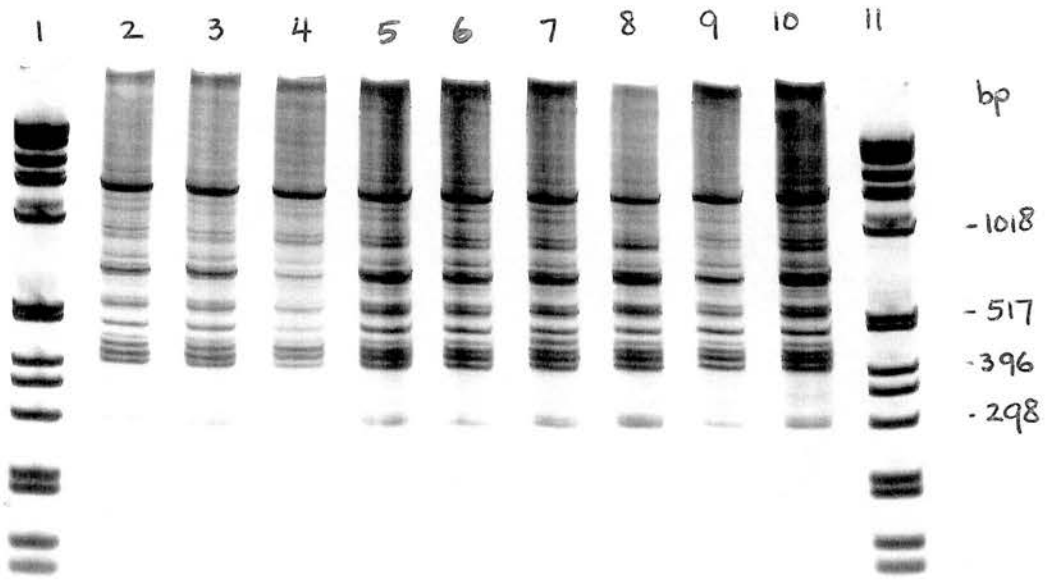
10
11
bp
1018
517
396
298
220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bp
1018
517
396
298

The RAPD pattern 1 that was generated by the primer GEN-056, consists of only one amplification product of BAKIT 381 (Track 2, **Figure 5.12**). This pattern differed from the second one by the presence of an intensely stained band at 480 bp; while the second pattern has an intense band at 493 bp. Three intensely stained bands at 430, 384 and 312 bp were present in all of the amplification products of all stocks studied. In general, the banding pattern generated by the primer GEN-056 showed unclear pattern in bands present between 500-1,000 bp size range and bands smaller than 300 bp.

5.3.4.3. DISCUSSION

The RAPD analysis applied in the study detected heterogeneity in the RAPD profiles of *T. evansi* stocks amplified with GEN-046 and -056. Some amplification products showed more intensely banded patterns and detected greater number of bands compared to others (for example, the banding patterns shown by the amplification products with primer GEN-054 in **Figure 5.11**). Inequality of DNA content in agarose blocks might cause the differences in band intensities and numbers. The 1/8 *T. evansi* DNA agarose blocks used in the present study were cut by hand. This may cause unequal size in each block cut and therefore unequal DNA content. The DNA quantity is an important factor that influences the RAPD analysis (Neto *et al.*, 1993, Steindel *et al.*, 1993), however, Kanmogne *et al.* (1996) stated that the quantity of DNA template was not critical below a certain level. Reproducibility in the RAPD pattern was achieved when the reaction was carried out using 5-10 ng DNA template. A higher amount of DNA, as high as 20 ng, prevented amplification of many bands (Kanmogne *et al.*, 1996). No amplification was observed when the amount of DNA was increased to 40 ng (Kanmogne *et al.*, 1996). However, reducing the DNA template below a critical level may have produced background bands that were not detectable previously in higher DNA concentration (Tyler *et al.*, 1997).

The *T. evansi* stocks amplified with the arbitrary primer GEN-046 (5'-GAGACGTCCC) showed good resolution in their banding patterns. The polymorphism in the banding pattern was detected in bands between 240 bp to 540 bp size range. The presence or absence of the unstable bands between this size range was then used for stock comparison.

Inconsistency of an overall RAPD fingerprint was detected by Bishop *et al.* (1993) who suggested that this could be due to the competition of the single primer binding at many sites in the genome. It was also suggested that the use of uncloned stock might vary the priming sites due to the variation in the sequence of different clones (Bishop *et al.*, 1993). The primer GEN-046 used in the study generated stable RAPD patterns in cloned and uncloned *T. evansi* stocks, in the same stocks prepared on a number of occasions and some stocks run at different times. The RAPD analysis carried out in this study had been found to be

useful for *T. evansi* stock comparison. It suggests, however, that the identical bands in the RAPD patterns do not necessarily represent an homologous amplified region; it only represents fragments of identical size that differ in respective locations within the genome (Petrie *et al.*, 1996).

This study used a set of 10-mer arbitrary primers with 70% GC content; two of the primers generated polymorphism in the amplification products of *T. evansi* stocks tested. The result of the study was different from that described earlier by Waitumbi and Murphy (1993) who did not find any polymorphism among *T. evansi* stocks tested using the single arbitrary primer ILO525 with 80% GC content. Williams *et al.* (1990) suggested that a minimum of 40% GC content in a 10-mer oligonucleotide is required to generate detectable levels of amplification products and a useful primer length is an oligonucleotide consisting of a minimum of nine bases. Shorter single primers (consisting of 5-8 bases) will generate a large numbers of bands and complicated banding patterns as shown in the DAF (DNA amplification fingerprinting), a type of RAPD analysis developed by Caetano-Anolles *et al.* (1991).

Intra-species differences generated by *T. congolense* amplified with the primer ILO525 was reported by Waitumbi and Murphy (1993). The primer did not distinguish between *T. b. brucei* and *T.b. rhodesiense*. However, *T.b. gambiense* had a different RAPD pattern from that of *T.b. brucei*. Dirie *et al.* (1993b) determined the DNA fingerprints of *T. vivax* using a set of 35 arbitrary primers with GC contents between 60% and 90%. Only one primer (ILO525: 5'-CGGACGTCGC) gave isolate-specific fingerprints for *T. vivax* stocks tested (Dirie *et al.*, 1993b) which could be separated into a Kenyan group and a group comprising other *T. vivax* stocks originating from Uganda, Nigeria, Gambia and Colombia. This study had shown the influence of G + C content in the primer and the sequence of the oligonucleotide in the RAPD analysis. Because of the clarity in the banding patterns shown by the amplification products and the ability to detect polymorphisms in the RAPD patterns, the single arbitrary primer, GEN-046, (5'-GAGACGTCCC) was then selected to amplify the DNA from the Indonesian *T. evansi* stocks available as agarose embedded DNA blocks.

5.4. COMPARISON OF *T. EVANSI* STOCKS BY RAPD ANALYSIS USING GENOSYS 046 PRIMER

5.4.1. INTRODUCTION

The random amplified polymorphic DNA (RAPD), initially developed by Williams *et al.* (1990) and Welsh and McClelland (1990), generates reproducible characteristic fingerprint patterns according to the DNA source and primer used. RAPD analysis has been applied on the

differentiation of inter- and intra-species of trypanosomes (Waitumbi and Murphy, 1993; Tibayrenc *et al.*, 1993). Inter-species differences were noted in the arbitrarily primed-PCR (AP-PCR) by Waitumbi and Murphy (1993) using primer ILO 525 which showed a characteristic band in the *T. evansi* stocks of 287 bp and 273 bp in *T. brucei*. It was also concluded that the AP-PCR banding patterns tested did not reveal polymorphism within *T. evansi* stocks. Intra-species differences, however, have been shown in *T. vivax* (Dirie *et al.*, 1993b) when amplified with the same primer, ILO525.

Steindel *et al.* (1993) used RAPD to examine the genomic structure of *Trypanosoma cruzi* isolates in comparison with the zymodeme patterns. The RAPD profiles of the stocks from a variety of zymodemes tested suggested that the parasites within the same zymodemes are more closely related than those from the others.

The present study has shown polymorphism in the RAPD patterns of the *T. evansi* stocks from Indonesia, which were tested. Four different RAPD patterns have been detected. The RAPD analysis was performed to amplify *T. evansi* originating from Indonesia in order to achieve more understanding of the epidemiology of the parasite in Indonesia based on the polymorphism of RAPD patterns.

5.4.2. MATERIALS AND METHODS

5.4.2.1. Trypanosome Stocks

Isolation and collection of the 80 *T. evansi* stocks from Indonesia used in this study, has been described in the previous chapter (Characterisation of *Trypanosoma evansi* by pulsed-field gel electrophoresis) (**Table 4.1**). *Trypanosoma evansi* stocks from Kenya (TREU 1810) and Brazil (TREU 2187) and *T. brucei* (TREU 2177) and *T. congolense* (TREU 2193) stocks were also subjected in the study.

5.4.2.2. RAPD Amplification and Detection

The RAPD amplification and detection of the banding patterns were carried out according to the standard procedures as described in the section 5.2.1.1.

5.4.2.3. RAPD Pattern Analysis

The band sizes were estimated by comparing the 1 kb standard size marker (Gibco, BRL) and determined using BioImage ® Whole Band Analyser computer software package (Millipore, USA). Only bands in the size range of 230-540 bp were compared and then grouped, based on the percentage of the number of common bands shared between the 2

stocks compared over the total number of uncommon bands present in both stocks as described in Section 3.3. Dendrogram analysis was generated from the comparison of each stock by the BioImage® Whole Band Analyser computer software package.

5.4.3. RESULTS

5.4.3.1. Characterisation of All T. evansi *Stocks from Indonesia*

Analysis on the 80 *T. evansi* stocks studied showed polymorphism in the RAPD patterns. Four different patterns were detected in products in the size range of 230-540 bp. Nine stable bands (540 , 510 , 500 , 480 , 440 , 374 , 330 , 310 , 280 bp) were observed. The bands at 420 bp and 240 bp are not always present. Representatives of stocks showing the RAPD patterns 1 to 4 are shown in **Figure 5.15A, B, C and D**. The band sizes detected in the amplification products characteristic of four RAPD patterns in **Figure 5.15** are presented in **Table 5.9**. Stock comparisons were restricted to bands below 540 bp. The bands above 550 bp were not included in the analysis because the distances travelled by bands >550 bp were not consistent and in some gels were not well separated.

Table 5.9. The band sizes of the amplification products detected in the four RAPD patterns generated by the GEN-046 primer.

Band No.	Band Size (bp)	RAPD pattern			
		1	2	3	4
1	540	+	+	+	+
2	510	+	+	+	+
3	500	+	+	+	+
4	480	+	+	+	+
5	440	+	+	+	+
6	420	+	-	+	-
7	374	+	+	+	+
8	330	+	+	+	+
9	310	+	+	+	+
10	280	+	+	+	+
11	240	-	+	+	-

Note: -: Bands absent
+: Bands present

Figure 5.15 A. A representative of *T. evansi* stocks belonging to the RAPD pattern 1 and 2. The stocks were amplified with 0.3 μM of primer Gen 046.

Track 1 and 12: 1 kb standard size DNA marker

Track 2: BAKIT 372, RAPD pattern 1

Track 3: BAKIT 427, RAPD pattern 1

Track 4: BAKIT 425, RAPD pattern 1

Track 5: BAKIT 371, RAPD pattern 2

Track 6: BAKIT 510, RAPD pattern 2

Track 7: BAKIT 380, RAPD pattern 2

Track 8: BAKIT 503, RAPD pattern 1

Track 9: BAKIT 504, RAPD pattern 1

Track 10: BAKIT 373, RAPD pattern 1

Track 11: BAKIT 375, RAPD pattern 1

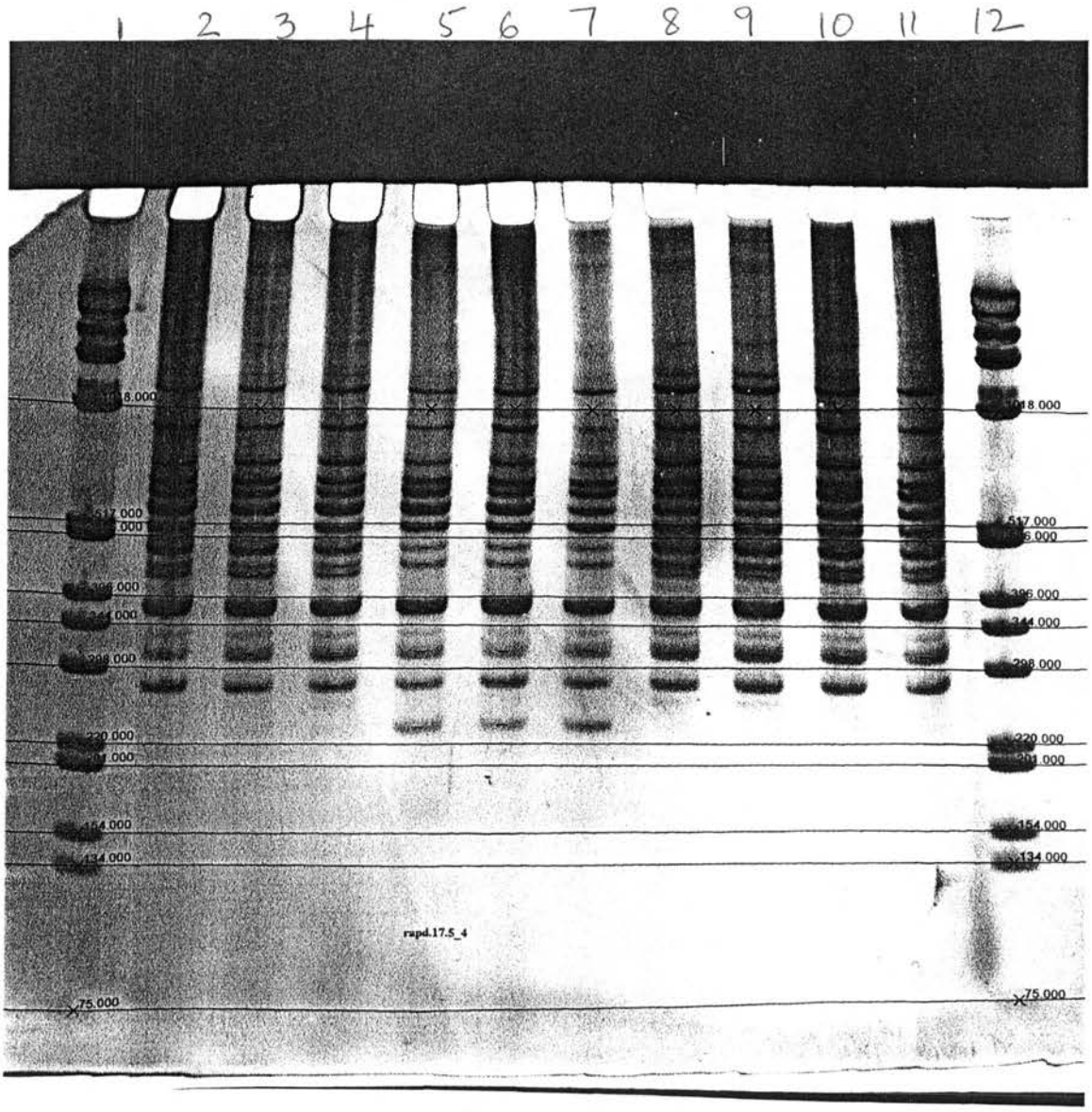

1 2 3 4 5 6 7 8 9 10 11 12
517.000
396.000
344.000
298.000
220.000
154.000
134.000
75.000
rapd.17.5_4

Figure 5.15 B. A representative of *T. evansi* stocks belonging to the RAPD pattern 1, 2 and 3. The stocks were amplified with 0.3 μM of primer Gen 046.

Track 1 and 12: 1 kb standard size DNA marker

Track 2: BAKIT 400, RAPD pattern 2

Track 3: BAKIT 508, RAPD pattern 1

Track 4: BAKIT 509, RAPD pattern 1

Track 5: BAKIT 382, RAPD pattern 3

Track 6: BAKIT 437, RAPD pattern 3

Track 7: BAKIT 514, RAPD pattern 1

Track 8: BAKIT 435, RAPD pattern 3

Track 9: BAKIT 423, RAPD pattern 3

Track 10: BAKIT 512, RAPD pattern 1

Track 11: BAKIT 394, RAPD pattern 2

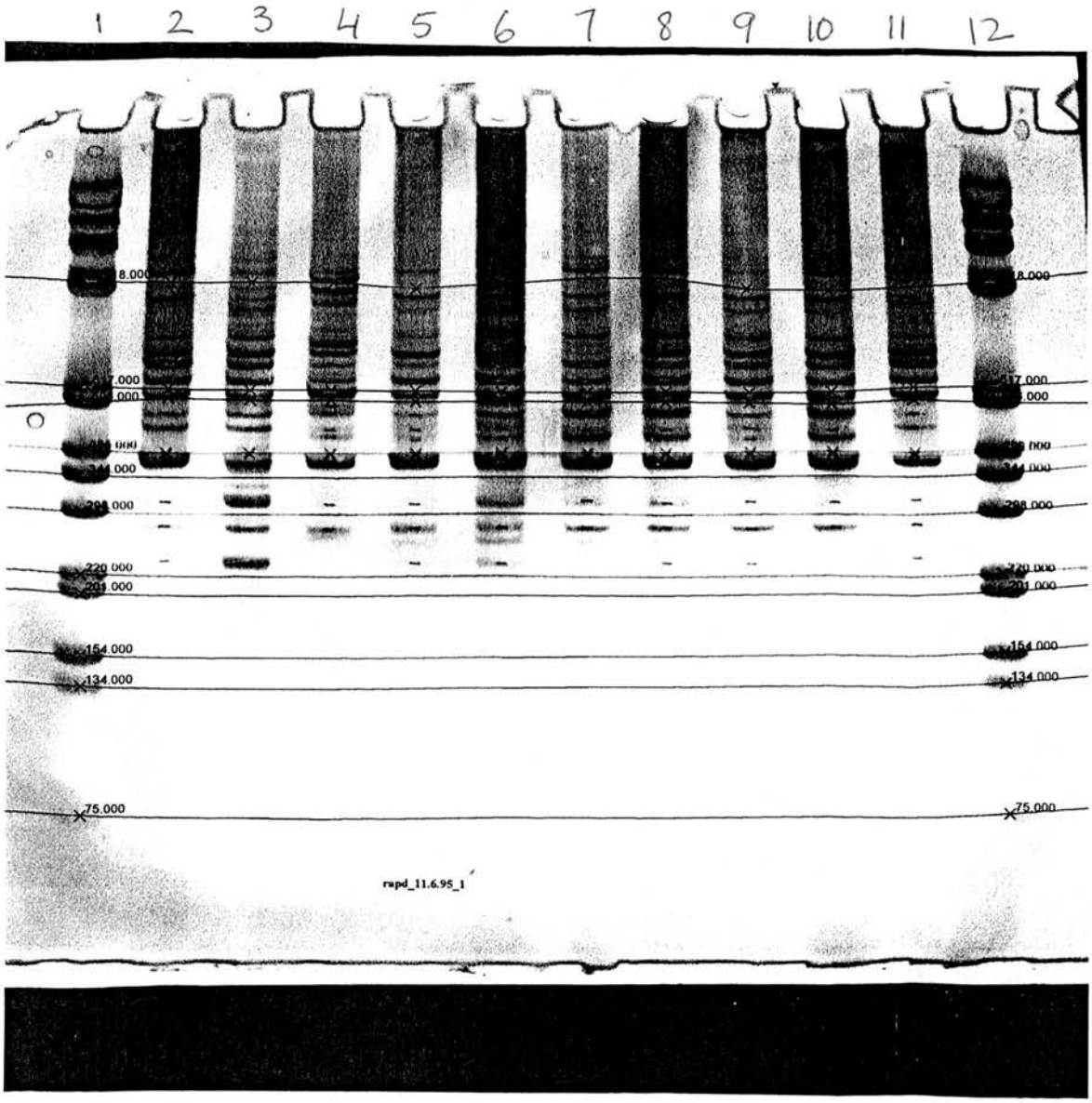

1 2 3 4 5 6 7 8 9 10 11 12
8.000
344.000
288.000
220.000
201.000
154.000
134.000
75.000
rapd_11.6.95_1

Figure 5.15 C. A representative of *T. evansi* stocks belonging to the RAPD pattern 2 and 4. The stocks were amplified with 0.3 μM of primer Gen 046.

Track 1 and 10: 1 kb standard size DNA marker

Track 2: BAKIT 411, RAPD pattern 4

Track 3: BAKIT 463, RAPD pattern 4

Track 4: BAKIT 445, RAPD pattern 4

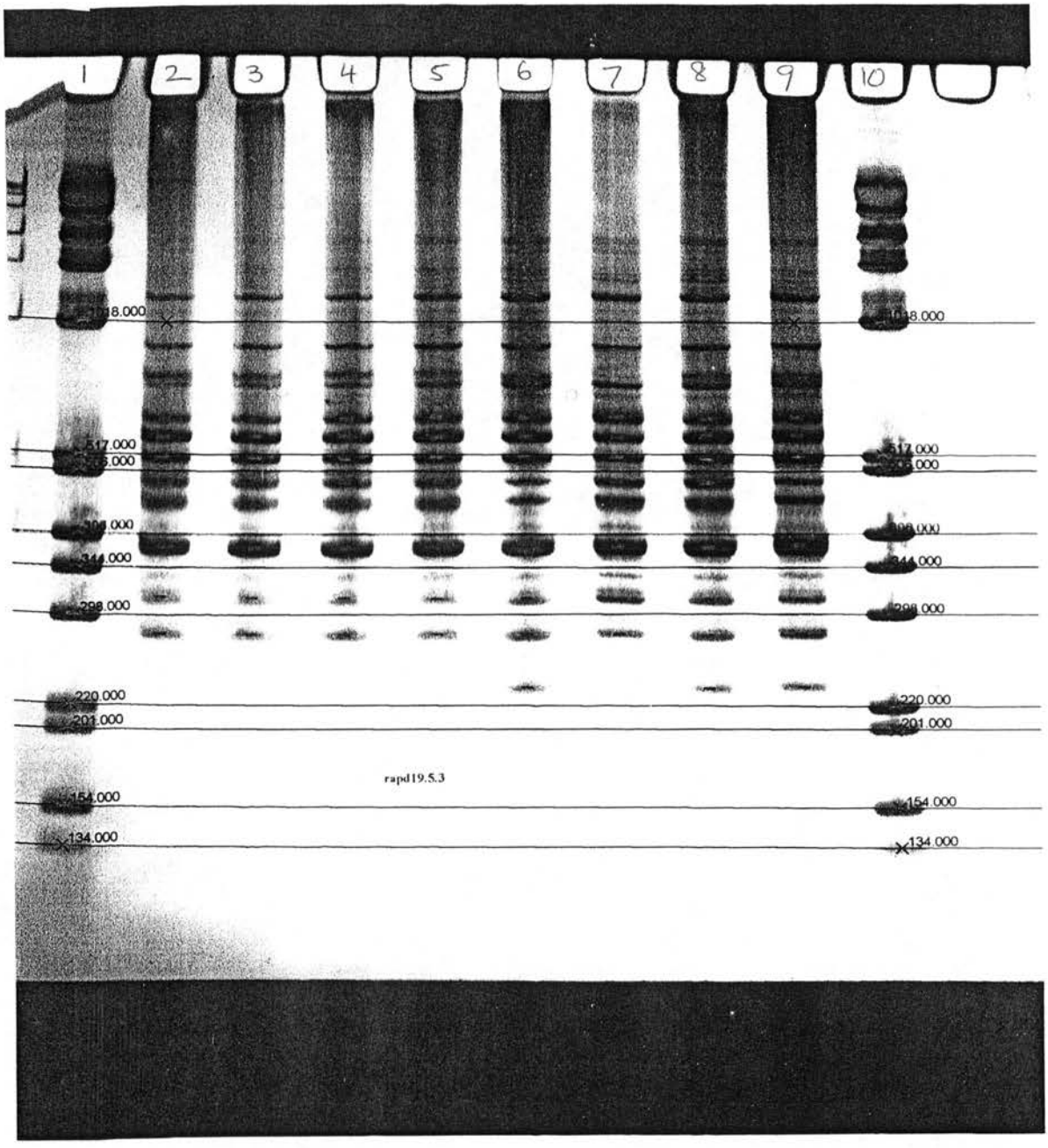
Track 5: BAKIT 446, RAPD pattern 4

Track 6: BAKIT 429, RAPD pattern 2

Track 7: BAKIT 511, RAPD pattern 4

Track 8: BAKIT 126, RAPD pattern 2

Track 9: BAKIT 386, RAPD pattern 2


1
2
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6
7
8
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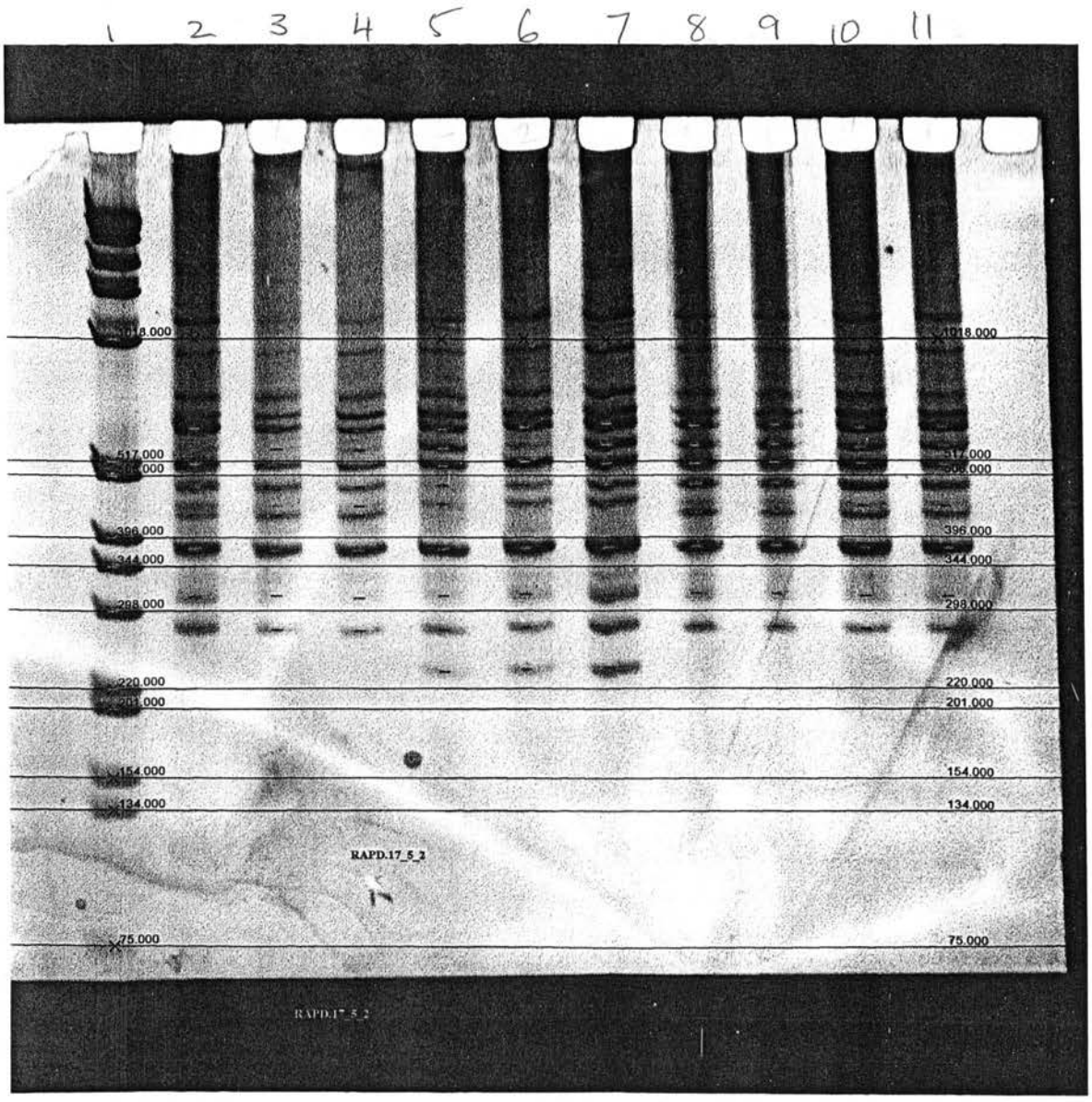
10
517.000
344.000
298.000
220.000
201.000
154.000
134.000
rapd19.5.3

Figure 5.15 D. A representative of *T. evansi* stocks belonging to the RAPD pattern 1 and 2. The stocks were amplified with 0.3 μM of primer Gen 046.

Track 1: 1 kb standard size DNA marker
Track 2: BAKIT 100, RAPD pattern 1
Track 3: BAKIT 312, RAPD pattern 1
Track 4: BAKIT 482, RAPD pattern 1
Track 5: BAKIT 296, RAPD pattern 2
Track 6: BAKIT 461, RAPD pattern 2
Track 7: BAKIT 371, RAPD pattern 2
Track 8: BAKIT 148, RAPD pattern 1
Track 9: BAKIT 254, RAPD pattern 1
Track 10: BAKIT 251, RAPD pattern 1
Track 11: BAKIT 403, RAPD pattern 1

Figure 5.16. Dendrogram analysis of RAPD patterns of the 80 *T. evansi* stocks from Indonesia


1 2 3 4 5 6 7 8 9 10 11
1018.000
517.000
396.000
344.000
298.000
220.000
201.000
154.000
134.000
75.000
RAPD.17_5_2

Results on the analysis of RAPD banding pattern similarity are shown in the dendrogram shown in **Figure 5.16**. The banding patterns shown by the 80 stocks were compared and assigned to one of the four RAPD patterns using a 50% grouping level.

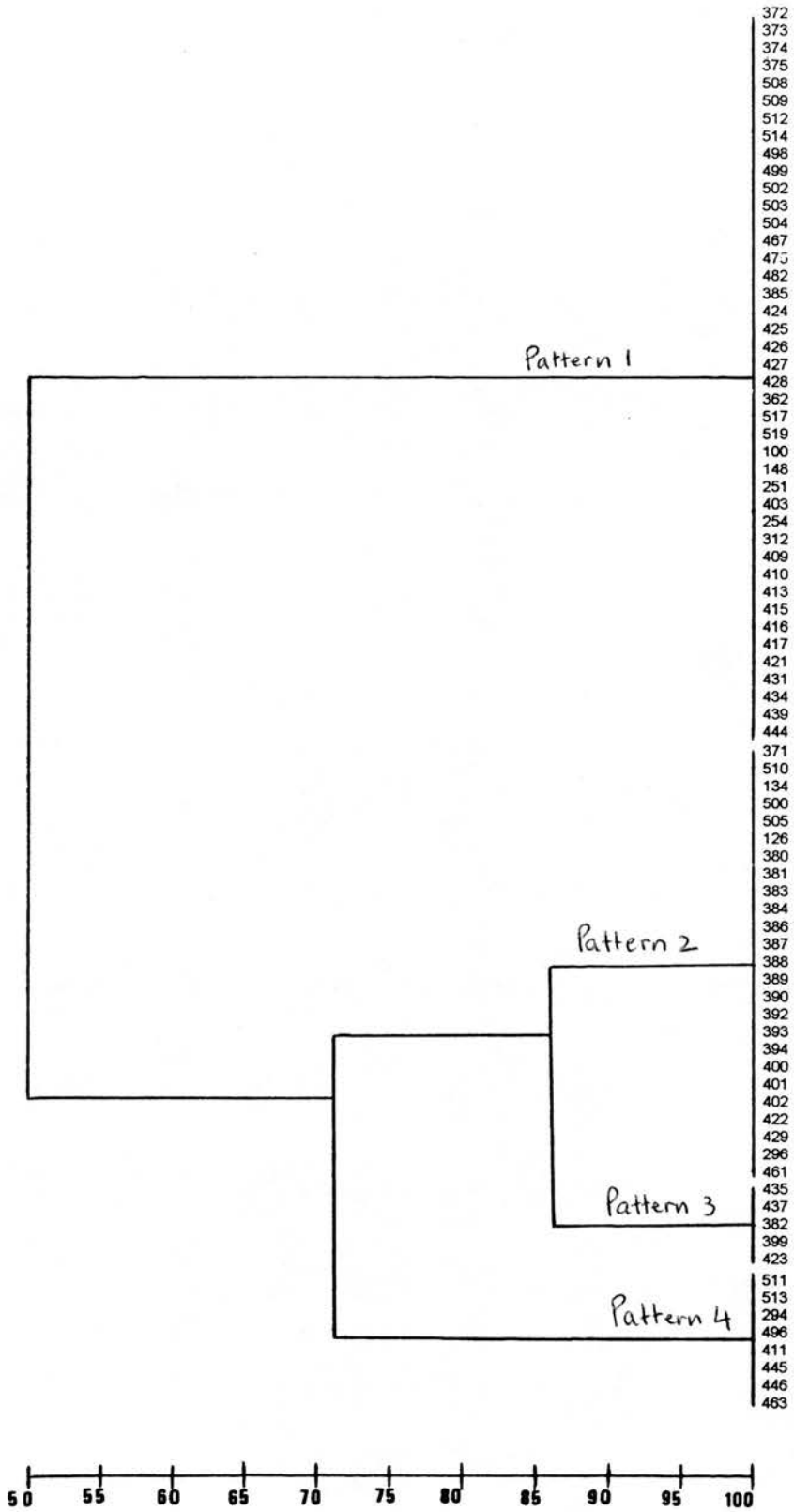
The RAPD pattern 1 is the predominant pattern in *T. evansi* stocks from Indonesia, with 54% of the stocks studied (42/80) belonging to this pattern. The RAPD pattern 1 was found in the stocks in each study area, being most commonly found in *T. evansi* stocks from Central Java (BAKIT 372, 373, 375, 374, 508, 509, 512, 514) and Lampung (BAKIT 409, 410, 413, 415, 416, 417, 421, 431, 434, 439, 444). This pattern was also found in stocks from West Java (BAKIT 498, 499, 502, 503, 504), East Java (BAKIT 467, 475, 482), Madura (BAKIT 362, 517, 519), North Sumatra (BAKIT 385, 424, 425, 426, 427, 428), North Sulawesi (BAKIT 100, 148, 254), South Sulawesi (BAKIT 251), South Kalimantan (BAKIT 403) and Aceh (BAKIT 312). The RAPD pattern 1 is characterised by the presence of a band at 420 bp and the absence of a band at 240 bp.

The presence of the RAPD pattern 2 in *T. evansi* stocks was not as widely distributed as pattern 1. The RAPD pattern 2 was found in many stocks collected from North Sumatra (BAKIT 380, 381, 383, 384, 386, 387, 388, 389, 390, 392, 393, 394, 400, 401, 402, 422, 429). Stocks from West Java (BAKIT 134, 505, 500), Central Java (BAKIT 371, 510), East Java (BAKIT 126), South Sulawesi (BAKIT 296) and South Kalimantan (BAKIT 461) were also belonged to this pattern. The RAPD pattern-2 is characterised by the absence of a band at 420 bp and the presence of a band at 240 bp.

The RAPD pattern 3 is characterised by the presence of both bands at 420 and 240 bp. The banding patterns revealed by the stocks belonging to pattern 3 seemed to be a combination of both RAPD patterns 1 and 2. The presence of *T. evansi* stocks belonging to this pattern is restricted to the stocks from Lampung (BAKIT 435 and 437) and North Sumatra (BAKIT 382, 399 and 423).

The RAPD pattern 4 differed from patterns 1, 2 and 3 by the absence of both bands at 240 bp and 420 bp. The presence of the stock belonging to this pattern is also restricted to the *T. evansi* stocks from Java (BAKIT 294/West Java, BAKIT 511/Central Java, 513/Central Java, 496/Central Java, BAIT 496/East Java) and Lampung (BAKIT 411, 444, 445 and 463).

The overall distribution of the RAPD patterns detected in the 80 *T. evansi* stocks from 10 regions in Indonesia is summarised in the map presented in **Figure 5.17**. The number of stocks belonging to each RAPD pattern in the 10 regions of Indonesia is presented in **Table 5.10**.


372
373
374
375
508
509
512
514
498
499
502
503
504
467
475
482
385
424
425
426
427
428
362
517
519
100
148
251
403
254
312
409
410
413
415
416
417
421
431
434
439
444
371
510
134
500
505
126
380
381
383
384
386
387
388
389
390
392
393
394
400
401
402
422
429
296
461
435
437
382
399
423
511
513
294
496
411
445
446
463
Pattern 1
Pattern 2
Pattern 3
Pattern 4
50
55
60
65
70
75
80
85
90
95
100
Percentage Similarity

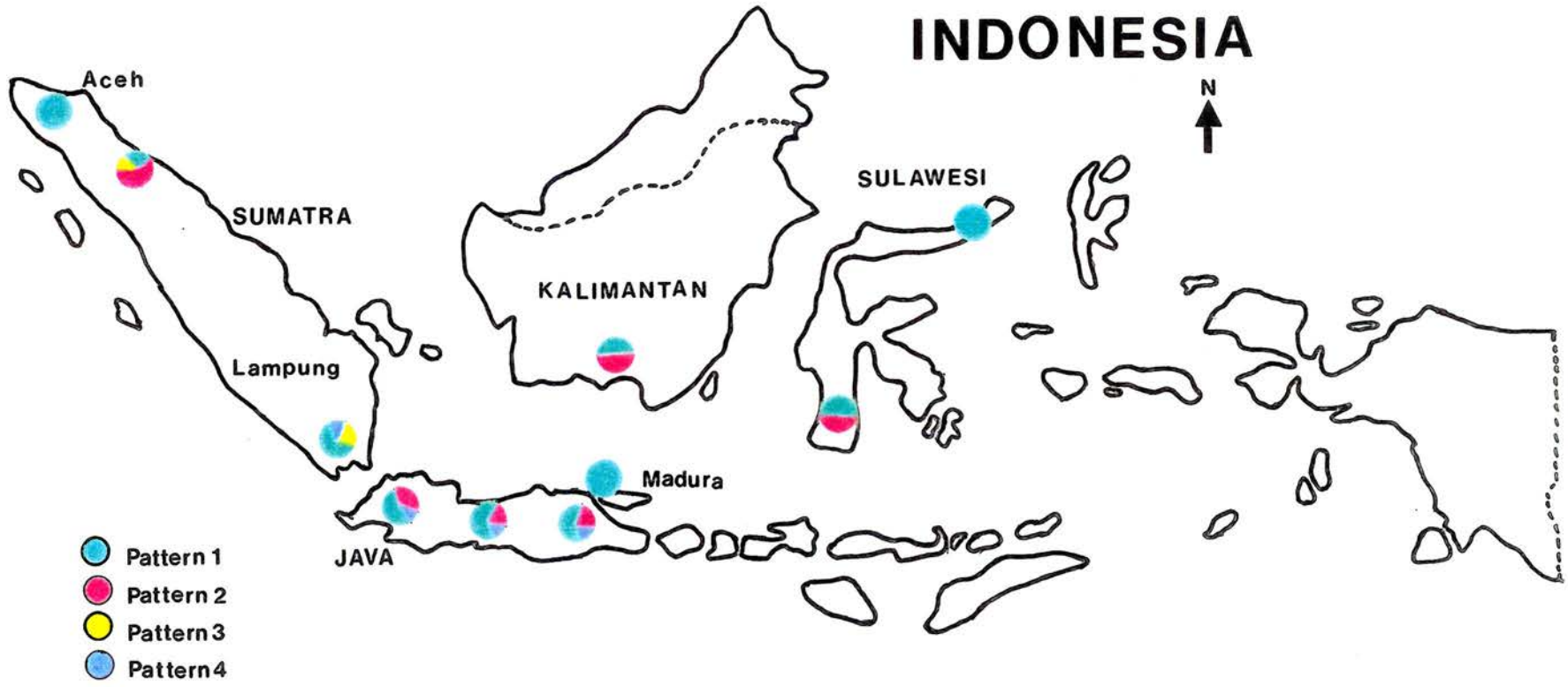


Figure 5.17. Map of Indonesia. Distribution of Random Amplified Polymorphic DNA patterns detected in *Trypanosoma evansi* stocks in Indonesia.

Table 5.10. The distribution of four RAPD patterns in 80 *T. evansi* stocks collected from 10 different regions in Indonesia.

Stock Isolation Locality	RAPD Pattern (No of stocks +)				Total no of samples
	1	2	3	4	
Central Java	8	2	-	2	12
Lampung	11	-	2	4	17
West Java	5	3	-	1	9
North Sumatra	7	16	3	-	26
East Java	3	1	-	1	5
Madura	3	-	-	-	3
North Sulawesi	3	-	-	-	3
South Kalimantan	1	1	-	-	2
South Sulawesi	1	1	-	-	2
Aceh	1	-	-	-	1
Total	43	24	5	8	80
(%)	(54)	(30)	(6.2)	(10)	

5.4.3.2. Characterisation of T. evansi *Stocks in Transported Buffaloes by RAPD Analysis*

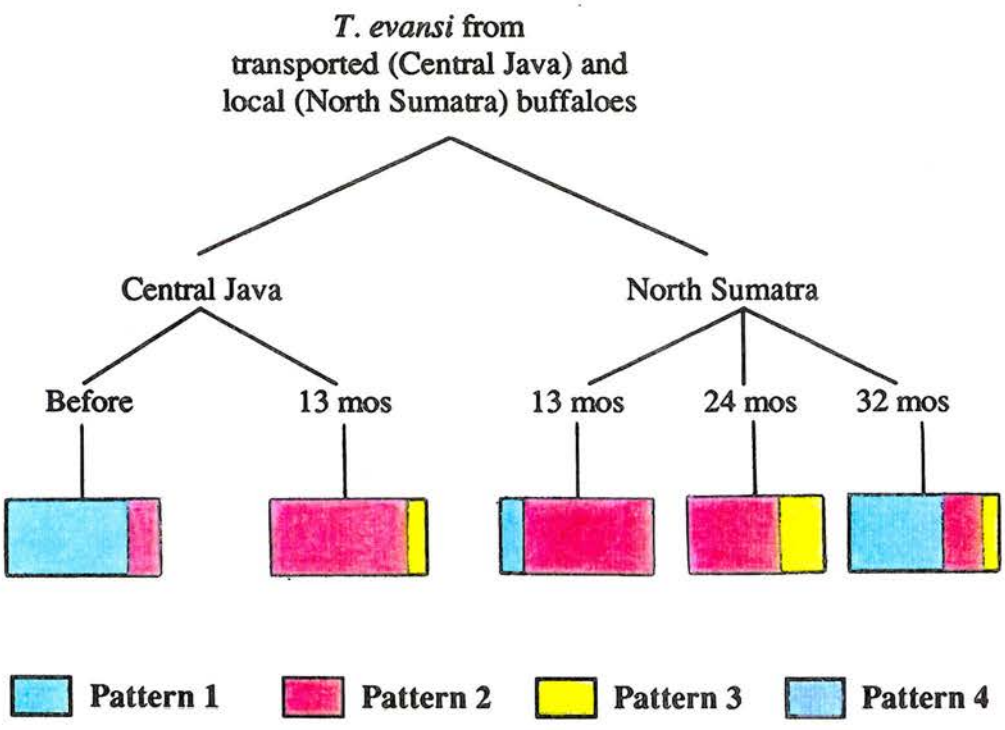
The presence of the RAPD patterns in *T. evansi* stocks collected from the transported buffaloes from Central Java to North Sumatra and from those collected from local buffaloes in North Sumatra are summarised in **Table 5.11** and the diagram presented in **Figure 5.18**

Table 5.11. Random polymorphic DNA patterns generated by the GEN-046 primer of *T. evansi* stocks collected from transported buffaloes from Central Java to North Sumatra and local buffaloes in North Sumatra.

Host Origin	RAPD Pattern (No of stocks)										
	Before transportation		Visit 1			Visit 2			Visit 3		
	1	2	1	2	3	1	2	3	1	2	3
Central Java	4	1	0	7	1	ND	ND	ND	ND	ND	ND
North Sumatra			1	5	0	0	2	1	5	2	1
Total	4	1	1	12	1	0	2	1	5	2	1

Note:
ND: Not Done
Visit 1 was carried out 13 months after the Central Java buffaloes arrived in North Sumatra.
Visit 2 was carried out 24 months after the Central Java buffaloes arrived in North Sumatra.
Visit 3 was carried out 32 months after the Central Java buffaloes arrived in North Sumatra.

Figure 5.18. Diagram of Random Amplified Polymorphic DNA patterns distribution of *Trypanosoma evansi* stocks isolated from transported buffaloes before and 13, 24 and 32 months after transportation.


T. evansi from
transported (Central Java) and
local (North Sumatra) buffaloes
Central Java
North Sumatra
Before
13 mos
13 mos
24 mos
32 mos
Pattern 1
Pattern 2
Pattern 3
Pattern 4

5.4.3.2.1. The RAPD patterns in *T. evansi* stocks from Central Java buffaloes

The RAPD patterns in *T. evansi* stocks from Central Java, before transportation, mainly belonged to the pattern 1 (4/5 stocks) and one stock belonged to pattern 2 (**Table 5.11**). Thirteen months after exportation to North Sumatra, the predominant pattern of *T. evansi* stocks isolated from the buffaloes was pattern 2 (7/8 stocks); with none of the stocks collected at this time belonging to pattern 1.

5.4.3.2.2. The RAPD patterns in *T. evansi* stocks collected from local (North Sumatra) buffaloes

The *T. evansi* stocks isolated from local buffaloes 13 and 24 months after transportation of the Central Java buffaloes (5/6 stocks and 2/3 respectively) mainly belong to pattern 2 (**Table 5.11**). However, 32 months after the transportation of Central Java buffaloes, the RAPD patterns detected in the stocks isolated from local buffaloes were more variable, with pattern 1 detected in 5/8 stocks; pattern 2 in 2/8 stocks and pattern 3 in 1/8 stocks.

5.4.3.4. Characterisation of T. evansi *Stocks in a Bali Cattle Feedlot in Lampung by RAPD Analysis*

A high degree of similarities in the RAPD patterns were observed in the stocks from Lampung, with one predominant pattern (Pattern 1) found in the stocks collected (11/17 stocks). Three RAPD patterns were found in the 17 stocks isolated from the feedlot cattle in Lampung. The RAPD patterns distribution found in *T. evansi* stocks from Lampung is summarised in **Table 5.12**. The presence of pattern 1 still predominated (5/9 stocks) in the Lampung stocks collected one month later (visit II). However, there was more variation in the RAPD patterns detected among the stocks collected at the second visit with 2/9 stocks each belong to patterns 3 and 4 (**Table 5.12**). Two *T. evansi* stocks (BAKIT 411 and 435) collected at two different times, at a month interval, from the same animal showed different RAPD patterns (patterns 4 and 3 respectively). Only one stock (pattern 4) was collected in the third visit.

Table 5.12. Random amplified polymorphic DNA patterns in *T. evansi* stocks isolated from a Bali cattle feedlot in Lampung. The *T. evansi* stocks were amplified with a single arbitrary primer GEN-046.

Visit No.	RAPD Pattern (No of stocks)		
	1	3	4
I	6	0	1
II	5	2	2
III	0	0	1
Total	11	2	4

Note:
Visit II was carried out 1 month after visit I
Visit III was carried out 3 months after visit II

5.4.3.5. Comparison of the RAPD Patterns of T. evansi *Stocks Originated from Indonesia, Kenya and Brazil with Patterns Shown by* T. brucei *and* T. congolense

The RAPD patterns of *T. evansi* stocks originated from Kenya, Brazil and Indonesia and *T. brucei* and *T. congolense* stocks are presented in **Figure 5.19**. The band sizes, which were estimated using the BioImage® Whole Band Analyser, of the *T. congolense*, *T. brucei* and *T. evansi* stocks studied were presented in **Table 5.13**. Differences in the banding patterns between the stocks studied were detected based on the presence and absence of the bands in each stock.

Figure 5.19. The Random Amplified Polymorphic DNA patterns in trypanosome stocks using primer GEN-046.

Tracks 1, 10: 1 kb size standard marker
Track 2: TREU 2193
Track 3: TREU 2177
Track 4: TREU 1810
Track 5: TREU 2187
Track 6: BAKIT 381
Track 7: BAKIT 424
Track 8: BAKIT 431
Track 9: BAKIT 399

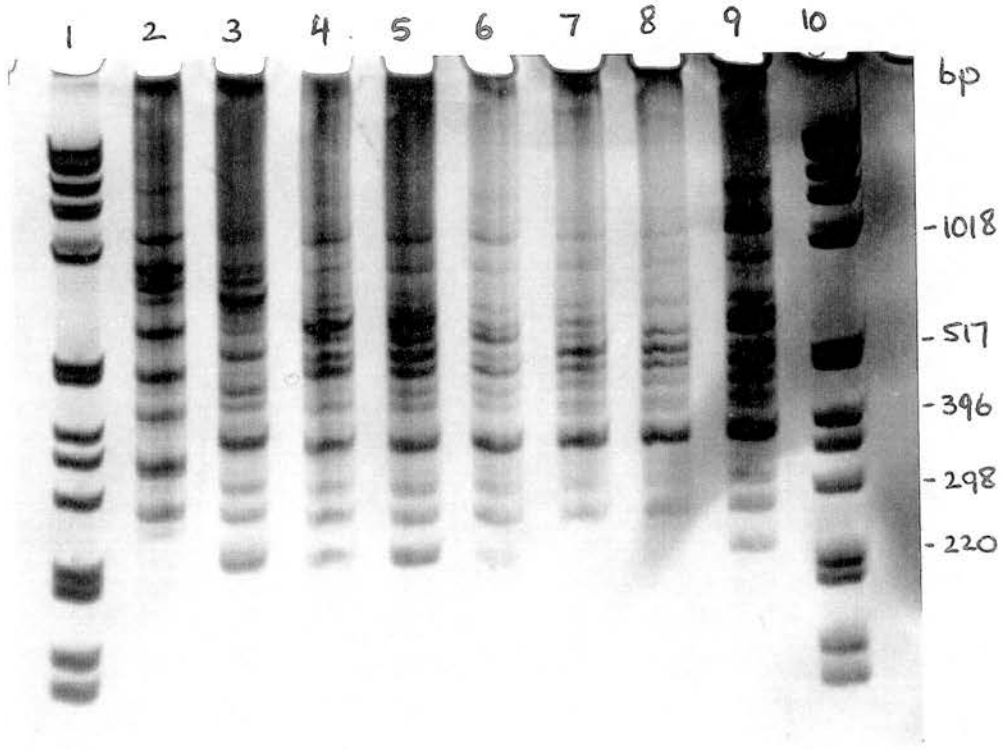

1
2
3
4
5
6
7
8
9
10
bp
1018
517
396
298
220

Table 5.13. Random amplified DNA patterns in *T. evansi* from Indonesia (4 stocks), Kenya (1), Brazil (1), *T. brucei* (1 stock) and *T. congolense* (1 stock) amplified by the GEN-046 primer.

Band No.	Band Size (bp)	*T. co*	*T. br*	*T. evansi* (BAKIT No)					
				Kenya	Brazil	399	381	424	431
1	1,100	+	+	+	+	+	+	+	+
2	850	++	+	+	+	+	+	+	+
3	800	-	+	-	-	-	-	-	-
4	725	++	-	-	-	-	-	-	-
5	655	+	++	+	+	-	-	-	-
6	620	-	±	+	+	+	+	+	+
7	570	+++	+	++	++	++	+	+	-
8	540	-	++	+	+	+	+	+	+
9	510	+++	-	+	+	+	+	+	+
10	500	-	-	+	+	+	+	+	+
11	480	±	+	+	+	+	+	+	+
12	440	-	-	+	+	+	+	+	+
13	420	+	-	-	-	+	-	+	+
14	374	-	+++	+++	+++	+++	+++	+++	+++
15	330	+++	-	-	-	±	±	±	±
16	310	-	+	+	+	+	+	+	+
17	280	++	+	+	+	+	+	+	+
18	260	±	-	-	-	-	-	-	-
19	240	-	+	+	+	+	+	-	-

Note:
T. br = *T. brucei*
T. co = *T. congolense*
- = band absent
Band intensity ranging from ± (faint) to +++ (very intense)

5.4.4. DISCUSSION

5.4.4.1. Indonesian Stocks

Compared to the pulsed-field gel electrophoresis (PFGE) used for karyotyping, which classified the 80 *T. evansi* stocks studied into 47 different karyotype patterns, the RAPD analysis produced 4 different patterns in the size range of 230-550 bp in the same stocks studied. The PFGE determines size classes of individual chromosomes, which vary due to chromosomal rearrangements (Van der Ploeg *et al.*, 1984a), while the RAPD analysis detects polymorphisms of specific DNA sequences in the genome.

The study carried out in the 80 *T. evansi* stocks has shown that the RAPD analysis distinguished the isolates according to their geographic location, which corresponds with results shown by karyotype analysis. Differences in the detection techniques between karyotyping and RAPD analysis have shown that identical karyotype patterns do not always show identical RAPD patterns. However, both techniques have shown evidence of stock isolation locality in *T. evansi* stocks collected from different regions of Indonesia. The study has shown that karyotyping has grouped *T. evansi* stocks at a finer level than RAPD analysis. For example, different karyotype patterns were observed in the stocks from Java and Lampung, whilst the RAPD analysis had recognised them as identical patterns.

Comparison of the two techniques, karyotyping and RAPD analysis, was carried out by Lun and Desser (1996b) in anuran trypanosomes. It was reported (Lun and Desser, 1996b) that most chromosome profiles of the anuran trypanosomes studied were in accordance with the polymorphism shown in RAPD patterns detected. The RAPD analysis carried out by Waitumbi and Murphy (1993), however, did not reveal polymorphism among *T. evansi* isolates from Kenya, which had been shown by karyotyping studies. The present study has shown that *T. evansi* stocks from Indonesia are more variable in the RAPD patterns. Polymorphisms in the RAPD banding patterns were observed among the Indonesian stocks studied using both GEN-046 and ILO525 primers.

The RAPD pattern analysis has shown the correlation between the patterns detected with the location from where the stocks were isolated. This was shown by the presence of a specific predominant pattern in an area, such as pattern 1 was predominant in stocks from Java and Lampung and, pattern 2, in stocks from North Sumatra. The presence of pattern 3, however, was only detected in Sumatra (Lampung and North Sumatra). It is not known yet whether this pattern is unique to Sumatra since there were few numbers of *T. evansi* stocks collected from areas other than Java, Lampung and North Sumatra. The presence of RAPD pattern 4 was only detected in stocks from Java and Lampung.

The variation of RAPD patterns among *T. evansi* stocks in Indonesia may be related to the geographical isolation, where all the stocks studied were collected from widely distributed areas in Indonesia. Transportation of livestock among the endemic areas, to improve livestock productivity in Indonesia, might also influence the RAPD patterns variation in *T. evansi* stocks. This probably will explain the wide spread of pattern 1 and 2 in most of the study areas, because the transportation of infected animals and the presence of the fly vector were also significant in Indonesia.

The RAPD analysis has the advantage over PFGE because it is simpler and shows less variation in banding patterns to be compared than the karyotyping study. The presence of high molecular size bands, between 550-1,100 bp size range, in the RAPD products, detected in some preparation of 5% polyacrylamide gel, however, were not consistent nor

well separated and therefore were unsuitable for band comparison. This may due to the presence of amplified non-specific bands causing difficulties in identifying the true polymorphisms in this size range. The RAPD analysis is different from the two-primer mediated PCR. Low stringency conditions are applied in the RAPD analysis allowing the single arbitrary primer to mismatch with its target sequence (Welsh and McClelland, 1990). It was also observed that changes in the running time of the electrophoresis have made the DNA fragments move different distances. The electrophoresis for the RAPD products detection was run at the same time, or when the bromophenol blue added in the samples had reached the bottom of the gel. However, for some reason a slight inconsistency in the DNA movements, as shown by the 1 kb standard marker, was observed. It was, therefore, decided that polymorphisms in the banding patterns were compared in the bands smaller than 550 bp, and these banding patterns were reproducible.

5.4.4.2. Transported Buffaloes

The transportation of animals from Java is restricted only to western part of the island (e.g. from Central Java to North Sumatra). The RAPD patterns of the *T. evansi* stocks collected from the transported buffaloes have shown variations in their banding pattern; this polymorphism was also shown by the stocks collected from local buffaloes. None of the stocks collected were isolated from the same animals. Pattern 1 was predominant in the stocks collected in Central Java. However, the predominant pattern changed to pattern 2 when the animals were sampled 13 months later in North Sumatra. This also predominated in the stocks collected from local (North Sumatra) buffaloes. This might indicate that the Central Java buffaloes were infected with the *T. evansi* stock that also infected most of the local buffaloes. It was also possible that the pattern 2- type *T. evansi* had coexisted in Central Java buffaloes but was undetectable by the RAPD analysis because it was not a predominant pattern. This finding was in accordance to those of the karyotype study. The karyotype group 1.3 was predominant in the *T. evansi* stocks isolated from the Central Java and local buffaloes 13 months after transportation.

A new pattern (pattern 3) appeared 13 months after the buffaloes arrived in North Sumatra and persisted in the stocks collected from local buffaloes, although in a small numbers. The RAPD pattern 3 has a banding pattern, which is a combination of patterns 1 and 2. The appearance of the RAPD pattern 3 might imply the presence of genetic recombination between patterns 1 and 2. However, evidence of a sexual stage in *T. evansi* has not been reported. The RAPD pattern 3, however, was only present in Sumatra island (Lampung and North Sumatra). It is not known yet whether this pattern is unique to the *T. evansi* stocks in Sumatra, however, it was shown previously (Chapter 4. Molecular characterisation of

Trypanosoma evansi by pulsed field gel electrophoresis) that the karyotype group 1.4 was only detected in *T. evansi* stocks isolated in North Sumatra.

Polymorphism in the RAPD banding patterns was also detected in *T. evansi* stocks isolated from local buffaloes during the study period. It was observed that the predominant RAPD pattern 2 in the stocks isolated from local buffaloes in the first and second visits changed to pattern 1 in the third visit. The changes in the RAPD patterns may be due to the genomic rearrangements associated with the antigenic variation that had been detected in the karyotype study shown by Van der Ploeg *et al.* (1984a).

5.4.4.3. Bali Cattle Feedlot in Lampung

The collection of *T. evansi* stocks in a feed lot in Lampung was carried out as a part of the study to evaluate the efficacy of treatment on body weight gain using suramin (Naganol, Bayer). Only one isolate was collected after treatment; this might be due to the elimination of *T. evansi* in treated animals or untreated animals have died because of the severity of infection. The parasite prevalence rates in the samples taken was higher in the second visit than in the first visit (Payne *et al.*, 1994a), treatment of animals were carried out after samples were taken in the second visit.

The similarities in the banding patterns of *T. evansi* collected from animals kept in the feed lot suggest that a *T. evansi* population is predominantly present in the area. This is also supported by previous results, which show a similarity in the karyotype patterns indicating that the same type infected the animals. It is not known whether the animals were already infected when they were brought in, or if they were infected when they came in to the area.

Two stocks isolated from the one animal in the first and second visits showed a different RAPD pattern, which is in agreement with the karyotyping that showed two different karyotypes in both stocks. The results supported the idea that it could have been different *T. evansi* populations infecting the same animal, in which the second populations appeared after the disappearance of the first population; or the animal had been super-infected with a different strain. It may also be due to the genomic rearrangements (Van der Ploeg *et al.*, 1984a) altering changes in the RAPD pattern, which had been suggested by Kanmogne *et al.* (1996).

5.4.4.4. Comparison between the RAPD Patterns Detected in T. evansi *Stocks from Indonesia, Kenya and Brazil and Patterns Detected in* T. brucei *and* T. congolense *Stocks*

The RAPD analysis carried out in the present study has also detected inter-species differences. The RAPD patterns shown by *T. evansi* stocks differed from those of *T. brucei* and *T. congolense*. The RAPD pattern shown by the *T. brucei* stock was similar to those of *T. evansi*, indicating the close relationships between the two species. The *T. congolense* stock tested, however, showed a distinct RAPD pattern from those of *T. evansi* and *T. brucei*.

This study has shown the intra-species variation in the RAPD patterns among *T. evansi* stocks collected from different countries. However, there were no differences in the RAPD banding patterns between *T. evansi* stocks from Kenya and Brazil. This may be caused by the primer used in the RAPD analysis not detecting polymorphism between the two stocks from Kenya and Brazil due to the similarity in their sequence. To confirm monomorphism in the RAPD patterns in *T. evansi* stocks from Kenya and Brazil more stocks and probably different single primer sets should be tested.

Dendrogram analysis placed all *T. evansi* stocks in the same cluster indicating close relationship among the stocks studied. This evidence could also support the theory of single origin of *T. evansi* as has been stated by Gibson *et al.* (1983) and Boid (1988) based on the isoenzyme studies. A single origin for *T. evansi* has also been supported by the finding of the homogeneity in the minicircle sequences of *T. evansi* from all over the world (Songa *et al.*, 1990; Ou *et al.*, 1991; Lun *et al.*, 1992b; Artama *et al.*, 1992).

5.5. RAPD ANALYSIS ON DNA TEMPLATE PREPARED FROM *T. EVANSI* STABILATES

5.5.1. INTRODUCTION

Analysis of the RAPD patterns on *T. evansi* stocks originating from 10 different regions in Indonesia has revealed polymorphism in the stocks tested. All of the samples for RAPD analysis were prepared from *T. evansi* DNA embedded in agarose used for karyotype study. The agarose embedded DNA was also used by Waitumbi and Murphy (1993) for their RAPD analysis. The alternative sample preparation for RAPD would be phenol extraction of DNA followed by ethanol precipitation (Sambrook *et al.*, 1989) as described by Majiwa, Maina, Waitumbi, *et al.* (1993); Steindel, *et al.* (1993; 1994); Stevens and Tibayrenc (1995). But these methods need a great number of parasites and are time consuming. In order to circumvent this problem, and to be able to test as many cryopreserved samples as possible, an experiment was carried out to examine the possibility of using DNA template straight from stabilates for RAPD analysis. The DNA for the analysis was prepared without prior phenol extraction/ethanol precipitation. The second part of the study was carried out to confirm the reliability of RAPD analysis on DNA prepared directly from trypanosome stabilates.

5.5.2. MATERIALS AND METHODS

5.5.2.1. Trypanosomes

One *T. evansi* stock (TREU 2225; originally BAKIT 374 isolated from a buffalo in Central Java) was used for preliminary work on the sample preparation. Cryopreserved trypanosome stabilates listed in **Table 5.14** stored at CTVM were used in the study. Eight *T. evansi* stocks originating from Indonesia, 5 stocks from Kenya, 10 stocks from Sudan, 1 stock each from Colombia and Nigeria and a *T. brucei* stock were included in the study. Additionally, an agarose embedded DNA of a *T. brucei* stock (TREU 2177) was also included in the study.

Table 5.14. Trypanosome stocks used in the RAPD analysis. The DNA samples were prepared directly from cryopreserved stabilates.

TREU	Isolation locality		Host	Species
	Country	Region		
1179	Colombia	Arauca	Horse	*T. evansi*
1902	Indonesia	Pekalongan, Central Java	Buffalo	*T. evansi*
1912	Indonesia	Bogor, West Java	Cattle	*T. evansi*
1949	Indonesia	Tuban, East Java	Cattle	*T. evansi*
1900	Indonesia	Tuban, East Java	Buffalo	*T. evansi*
1908	Indonesia	Bekasi, West Java	Buffalo	*T. evansi*
1913	Indonesia	Sukabumi, West Java	Cattle	*T. evansi*
1914	Indonesia	Minahasa, North Sulawesi	Cattle	*T. evansi*
1915	Indonesia	Bogor, West Java	Buffalo	*T. evansi*
1950	Kenya	?	?	*T. evansi*
1777	Kenya	Kissima, Rumuruti	Camel	*T. evansi*
1778	Kenya	Kissima, Rumuruti	Camel	*T. evansi*
1789	Kenya	Kissima, Rumuruti	Camel	*T. evansi*
1787	Kenya	Olmaisor, Rumuruti	Camel	*T. evansi*
2066	Nigeria	Mongonu	Camel	*T. evansi*
1450	Sudan	Soba, Khartoum	Camel	*T. evansi*
1471	Sudan	?	Camel	*T. evansi*
1898	Sudan	Hadaliya, Kassala	Camel	*T. evansi*
1445	Sudan	Hadaliya, Kassala	Camel	*T. evansi*
1418	Sudan	Kassala	Camel	*T. evansi*
1412	Sudan	Aroma, Kassala	Camel	*T. evansi*
1448	Sudan	Khartoum	Camel	*T. evansi*
1452	Sudan	Khartoum	Camel	*T. evansi*
1449	Sudan	Khartoum	Camel	*T. evansi*
1419	Sudan	Tendelti, Blue Nile Province	Camel	*T. evansi*
2058	?	?	?	*T. brucei*

5.5.2.2. Sample Preparation

The sample preparation was carried out using an InstaGene Matrix® according to the procedures recommended by BioRad, UK. The trypanosome stabilate stored in a capillary tube was taken from a liquid nitrogen container and thawed at room temperature in a universal tube containing distilled water. The content of the capillary tube, ~ 50 μl of the trypanosome infected blood, was expelled into a 0.5 ml microcentrifuge tube and kept on ice. To optimise the conditions for sample preparation, different volumes of the sample (5, 10 and 20 μl and the whole content of the capillary) were taken out of the capillary. Each volume was distributed into 1.5 ml microcentrifuge tubes, then 1 ml of sterile deionised water (18.2 mΩ) was added into each tube. The mixture was homogenised by inverting the tubes several times and incubation of the mixture was carried out at room temperature for 15 minutes. Centrifugation of the tubes was carried out at 10,000 - 12,000 rpm in a microcentrifuge (Hawksley, England) for 2-3 minutes. After the centrifugation, the supernatant was removed carefully without disturbing the pellet, leaving approximately 20-30

μl of the supernatant. The InstaGene Matrix® (BioRad, UK) was stirred to homogenise the content and, using a large-bore pipette tip, a volume of 200 μl of the matrix was added to the pellet in each tube. Incubation of the mixture was carried out at 56^0 C in a waterbath for 15 minutes followed by vortexing at high speed for 10 seconds. The trypanosome DNA-matrix mixture was then boiled for 8 minutes and vortexed at high speed for 10 seconds. Centrifugation was carried out at 10,000 - 12,000 rpm using a microcentrifuge (Hawskley, England) for 2-3 minutes. The supernatant was used at a volume of 10 μl per 50 μl PCR reaction and the remainder can be stored at -20^0 C. The remaining frozen supernatant can be used after thawing, 10 seconds vortexing and centrifugation at 12,000 rpm for 10 seconds. The analysis also included cryopreserved uninfected mouse blood treated and not treated with InstaGene™ Matrix.

5.5.2.3. RAPD Amplification and Detection

The RAPD amplification and detection were carried out according to the standard procedures described in the section 5.2.1.1.

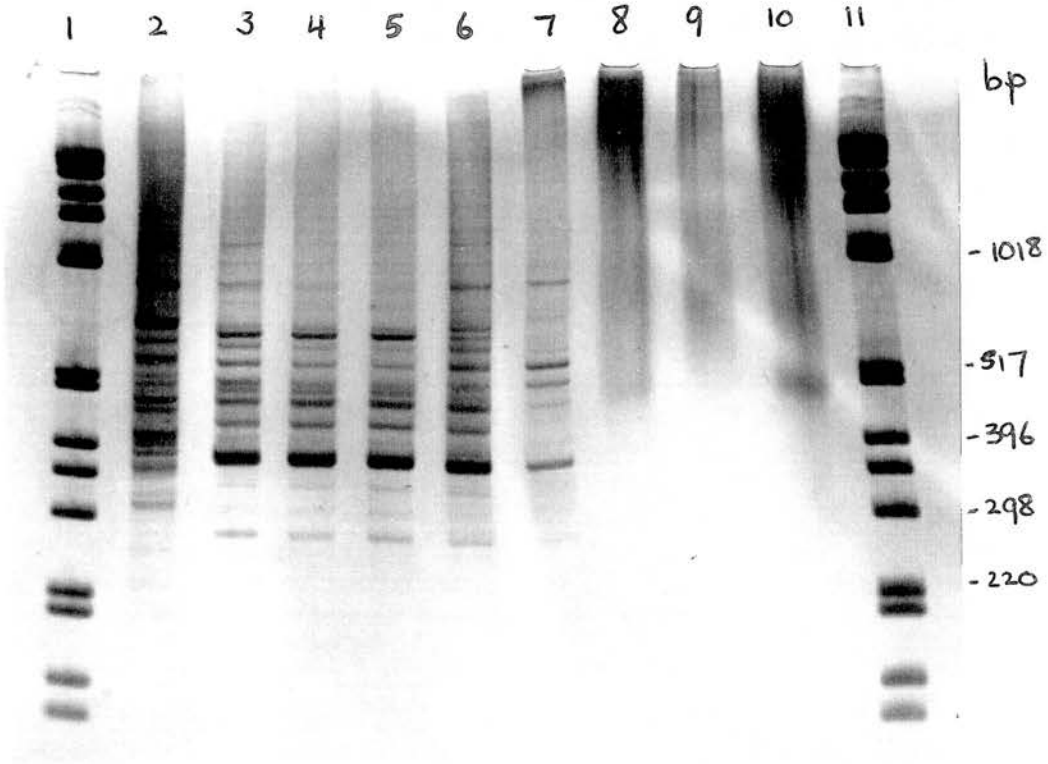
5.5.3. RESULTS

The RAPD patterns of the treated *T. evansi* stabilate and cryopreserved uninfected mouse blood to extract the DNA for RAPD analysis were shown in **Figure 5.20**. Samples of trypanosome stabilate and cryopreserved normal mouse blood without prior treatment with InstaGene® Matrix and the agarose embedded DNA were also included in the test.

The banding patterns shown by the *T. evansi* DNA prepared from treated stabilate and the agarose embedded DNA are similar to each other, and all belonged to the RAPD pattern 1. The banding pattern shown by the RAPD product generated by the agarose embedded *T. evansi* DNA was weaker compared to those shown by the *T. evansi* stabilate DNA amplification products. There were no differences in the banding patterns among the *T. evansi* DNA prepared from stabilate of different sample volumes: 5, 10 or 20 μl (Tracks 3, 4 and 5 in **Figure 5.20**). The banding pattern of the RAPD product generated by the whole *T. evansi* stabilate prepared DNA (Track 6 in **Figure 5.20**), however, showed more bands in high molecular size (above 540 bp) than those products generated by the lesser trypanosome stabilate content (Track 3-5). It was concluded that 5-10 μl of *T. evansi* stabilate content and processed using the InstaGene® Matrix (BioRad, UK) was sufficient for RAPD analysis DNA template. The banding pattern of the RAPD product generated by an agarose DNA sample of the same stock (Track 7 in **Figure 5.20**), however, did not show the same banding pattern with those of the samples prepared directly from the stabilate (Track 3-5 in **Figure 5.20**).

Figure 5.20. The Random Amplified Polymorphic DNA patterns of the *Trypanosoma evansi* DNA prepared directly from cryopreserved stabilates.

Tracks 1, 11: 1 kb standard DNA size marker
Track 2: Cryopreserved uninfected mouse blood processed using the InstaGene® Matrix
Track 3: DNA prepared from 5 μl previously cryopreserved stabilate, processed using the InstaGene® Matrix
Track 4: DNA prepared from 10 μl previously cryopreserved stabilate, processed using the InstaGene® Matrix
Track 5: DNA prepared from 20 μl previously cryopreserved stabilate, processed using the InstaGene® Matrix
Track 6: DNA prepared from 50 μl previously cryopreserved stabilate, processed using the InstaGene® Matrix
Track 7: DNA from 1/8 agarose block
Tracks 8,10: Unprocessed *Trypanosoma evansi* cryopreserved stabilate
Track 9: Unprocessed normal mouse blood previously cryopreserved in liquid Nitrogen.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None of the untreated samples showed any bands, with a smear of DNA seen in both samples (Tracks 8-10 in **Figure 5.20**). An unresolved banding pattern between 220-1,000 bp size range was detected in the RAPD product generated by the processed uninfected cryopreserved mouse blood, but this pattern was different from those of the amplified stabilate DNA. The RAPD pattern shown by the RAPD product of the mouse blood DNA is complicated and not well resolved.

5.5.3.1. The RAPD Patterns Generated by Trypanosome DNA Prepared Directly from Cryopreserved Stabilates

The RAPD patterns of the DNA extracted from stabilate blood of 8 Indonesian *T. evansi* stocks revealed polymorphism (**Figure 5.21**) although they showed different intensities in their banding patterns. The RAPD products generated by stocks TREU 1949 and 1914 (tracks 4 and 8 in **Figure 5.21**) showed more intense banding patterns compared to the others. RAPD banding patterns of stocks TREU 1912, 1908, and 1915 (tracks 3, 6 and 9 respectively in **Figure 5.21**) did not show clear banding patterns. The RAPD products generated by stocks TREU 1902 and 1900 (tracks 2 and 5 in **Figure 5.21**) showed similar pattern to that previously described as pattern 2 (Section 5.4.3.1). The amplification product of TREU 1913 (track 7 in **Figure 5.21**) showed similar banding pattern to the previously described in Section 5.4.3.1. as RAPD pattern 4.

The RAPD patterns of the 10 *T. evansi* stocks from Sudan and a stock from Nigeria were presented in **Figure 5.22** with different banding pattern intensity observed. A high degree of similarities in the RAPD patterns was detected in the *T. evansi* stocks from Sudan. The band sizes of the trypanosome stocks studied were summarised in **Table 5.15**. The RAPD pattern in the Sudanese stocks was similar to that of pattern 2 in the Indonesian stock with an additional band at 260 bp and the absence of a band at 470 bp in the Sudanese stocks. The *T. evansi* stock from Nigeria had a different pattern to those of the Sudanese stocks by the absence of bands at 450 bp and 310 bp and the presence of a band at 410 bp.

Figure 5.21. The Random Amplified Polymorphic DNA patterns in *Trypanosoma evansi* stocks from Indonesia amplified with primer GEN-046. The samples were prepared directly from cryopreserved stabilates using the InstaGene® Matrix.

Tracks 1, 10: 1 kb DNA standard size marker.
Track 2: TREU 1902 (BAKIT 007; Pattern 2)
Track 3: TREU 1912
Track 4: TREU 1949
Track 5: TREU 1900 (BAKIT 094; Pattern 2)
Track 6: TREU 1908
Track 7: TREU 1913 (BAKIT 097; Pattern 4)
Track 8: TREU 1914
Track 9: TREU 1915

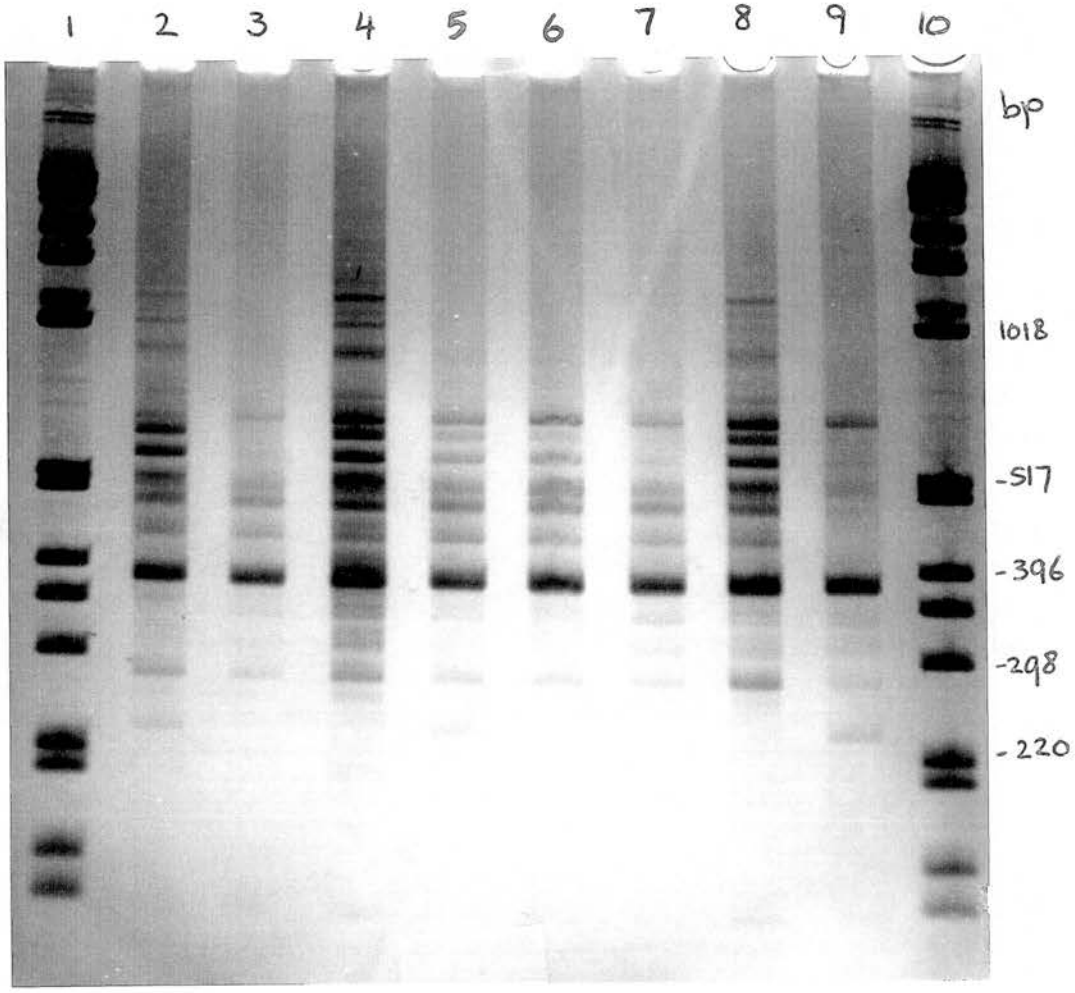
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Figure 5.22. The Random Amplified Polymorphic DNA patterns in *Trypanosoma evansi* stocks from Sudan and Nigeria amplified with the single arbitrary primer GEN-046. The samples were prepared directly from cryopreserved stabilates using the InstaGene® Matrix.

Tracks 1, 10: 1 kb DNA standard size marker.
Track 2: TREU 1450, Sudan
Track 3: TREU 1471, Sudan
Track 4: TREU 1898, Sudan
Track 5: TREU 1445, Sudan
Track 6: TREU 1418, Sudan
Track 7: TREU 1412, Sudan
Track 8: TREU 1448, Sudan
Track 9: TREU 1452, Sudan
Track10: TREU 1449, Sudan
Track11: TREU 1419, Sudan
Track12: TREU 2066, Nigeria

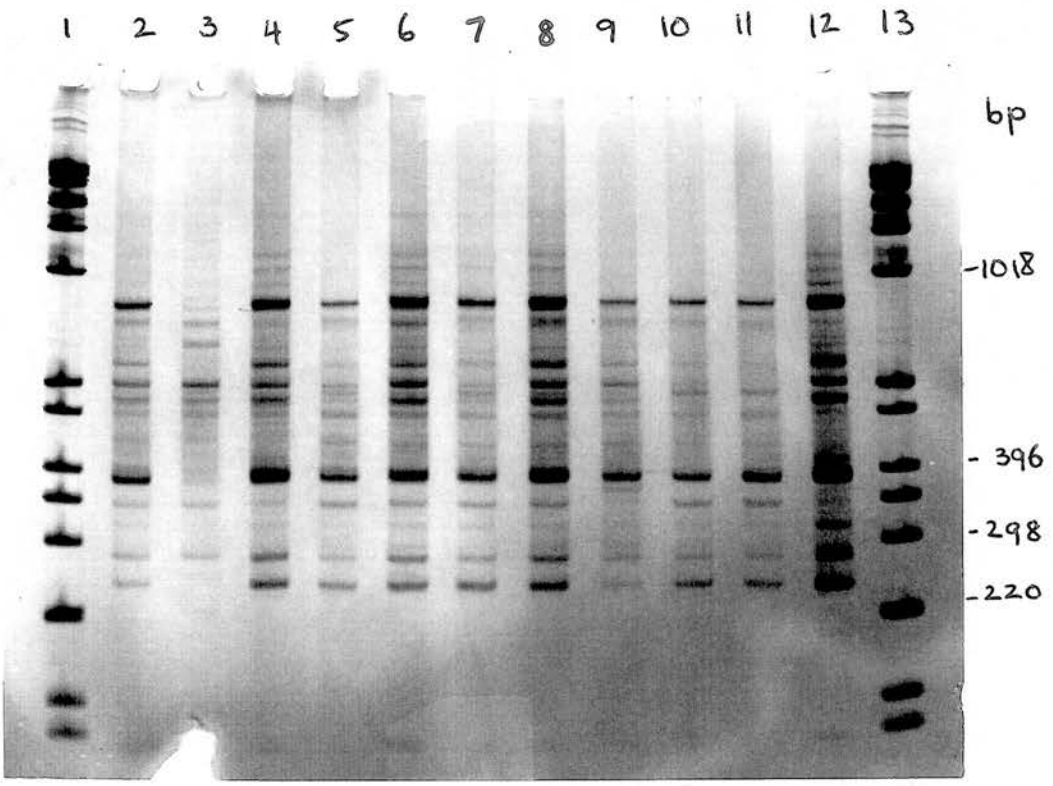
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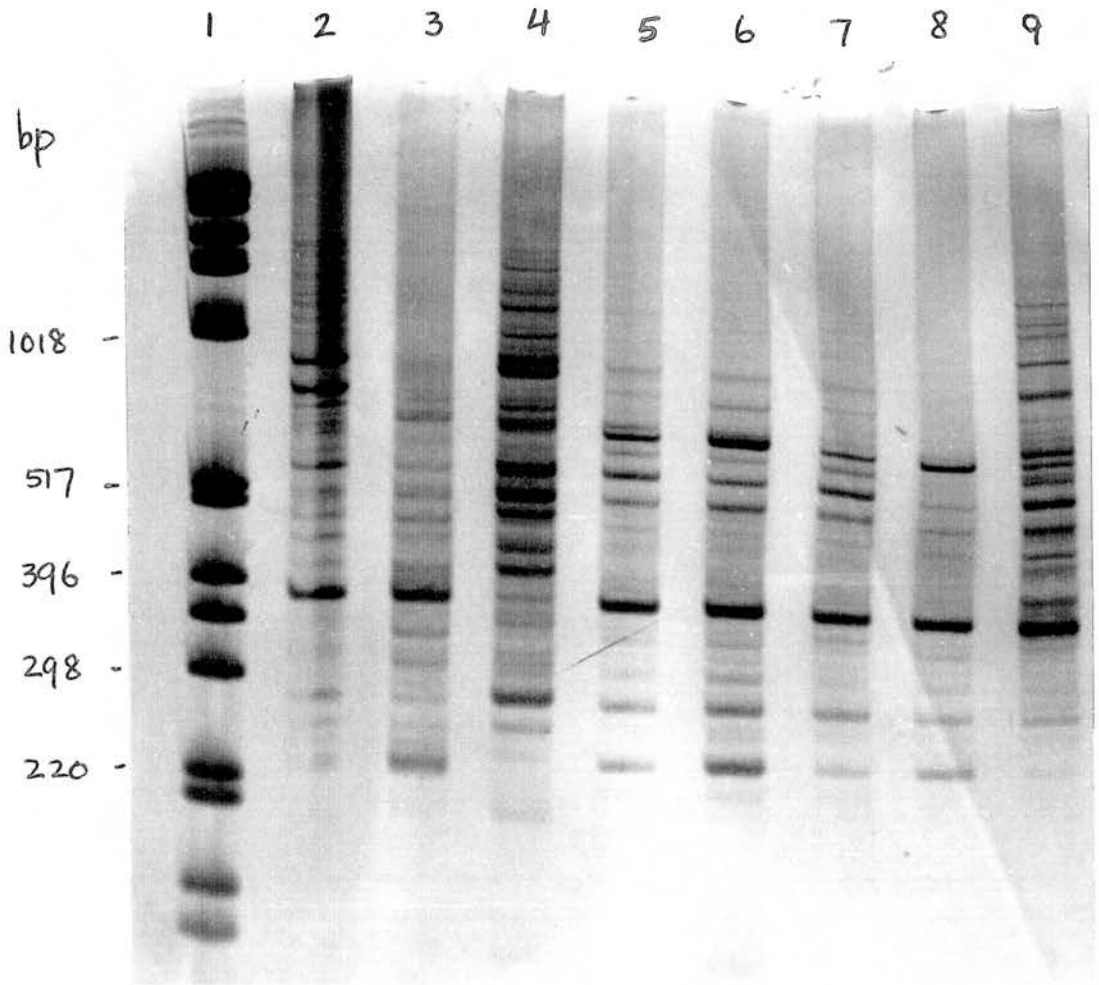
Table 5.15. A summary of the results on the RAPD patterns of *T. evansi* stocks isolated in Colombia (1), Indonesia (8), Kenya (5), Nigeria (1) and Sudan (10) amplified with the primer GEN-046. The DNA was prepared directly from cryopreserved stabilates.

Band No.	Band Size (bp)	Presence (+)/ Absence (-) of bands						
		Indonesia		Sudan	Kenya	Nigeria	Colombia	*T. brucei*
		P 2	P 4					
1	540	+	+	+	+	+	+	+
2	510	+	+	+	+	+	+	+
3	500	+	+	+	+	+	+	+
4	480	+	+	-	+	-	+	+
5	440	+	+	+	+	-	+	+
6	420	-	-	-	-	-	-	+
7	400	-	-	-	-	+	+	+
8	370	+	+	+	+	+	+	+
9	330	±	±	+	+	+	-	+
10	310	+	+	+	+	-	-	+
11	280	+	+	+	+	+	+	+
12	260	-	-	+	-	+	-	+
13	240	+	-	+	+	+	-	+

The RAPD patterns of *T. brucei* and *T. evansi* stocks from Kenya and Colombia are presented in **Figure 5.23**. The polyacrylamide gel electrophoresis separated 12 bands in the 1 kb DNA standard marker between 134-2036 bp size range. Four *T. evansi* stocks from Kenya displayed identical RAPD patterns and one stock seemed to have a very different banding pattern compared to the rest *T. evansi* stocks included in the analysis. The RAPD pattern in the *T. evansi* stocks from Kenya showed 10 bands in the size range of 230-550 bp, which is similar to the RAPD pattern 2 detected in the Indonesian stocks. The *T. evansi* stock from Colombia showed a different banding pattern compared to those of the stocks from Indonesia, Kenya, Sudan and Nigeria. The 2 *T. brucei* stocks had identical banding patterns with 13 bands in the size range of 230-550 bp.

Figure 5.23. The Random Amplified Polymorphic DNA patterns in *Trypanosoma evansi* and *Trypanosoma brucei* stocks using primer GEN-046. All samples, except TREU 2177, were prepared directly from cryopreserved stabilates using InstaGene® Matrix.

Track 1: 1 kb DNA standard size marker
Track 2: TREU 2177; *T. brucei*
Track 3: TREU 2058; *T. brucei*
Track 4: TREU 1950; Kenya
Track 5: TREU 1977, Kenya
Track 6: TREU 1778, Kenya
Track 7: TREU 1789, Kenya
Track 8: TREU 1787, Kenya
Track 9: TREU 1179; Colombia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5.5.4. DISCUSSION

RAPD banding patterns shown by the amplification products generated from stabilate-prepared DNA were different from that of the agarose embedded DNA. The agarose embedded DNA offered a better quality of DNA because it was prepared from trypanosomes that had been purified from the blood cells. The *T. evansi* DNA embedded in agarose had been used by Waitumbi and Murphy (1993) as the DNA template in their RAPD analysis using the ILO 525 arbitrary primer. It was suggested that purification of the parasites from host nuclei is necessary for AP-PCR or RAPD analysis (Waitumbi and Murphy, 1993), as the host DNA could interfere with the analysis of RAPD banding patterns.

The present study, however, has indicated that DNA prepared directly from cryo-stabilates can be used for RAPD analysis in substitution of the agarose embedded DNA. Treatment of the parasitaemic blood in the stabilate with InstaGene® Matrix (BioRad, UK) has made the procedures for sample preparation simpler. Approximately 10 μl out of 50 μl of the trypanosome stabilate contained in a single capillary tube is enough for the analysis. However, the number of trypanosomes in each stabilate is different, which may influence banding pattern intensity.

The banding patterns exhibited by *T. evansi* DNA samples prepared from stabilates were not clear in some preparations. This might be due to the differences in the number of trypanosomes contained in the stabilate. The presence of blood cells which are not completely lysed might also influence the number of bands in the RAPD analysis causing the unspecific binding with the host cells as the RAPD analysis uses a short single oligonucleotide as primer. The study has suggested that complete lysing of the blood cells is necessary when trypanosome stabilates are used as DNA template for RAPD analysis. This problem did not appear when the agarose embedded DNA was used as a template as it was prepared from purified trypanosomes from the host cells. In the RAPD analysis, the DNA was denatured at 94^0 C and then cooled at 40^0 C for primer annealing and the extension of the amplified product was carried out at 72^0 C. The presence of the agarose in the DNA template does not affect the amplification reaction, as the agarose remains soluble at 37^0 C.

The influence of PSG in the PCR reaction for *T. evansi* was reported by Wuyts *et al.* (1994). It was reported that 15 μl of PSG, containing 14.5 mM PO_4^{2-}, which is approximately seven-fold excess of PO_4^{2-} over free Mg^{++}, in 50 μl of PCR reaction completely inhibited the DNA amplification reaction. The Mg^{++} binds stoichiometrically to deoxynucleoside triphosphates, which is necessary for the DNA polymerase reaction. It was further suggested (Wuyts *et al.*, 1994) that the maximum two-fold ratio of PO_4^{2-} against Mg^{++} did not have significant effect on the PCR amplification reaction. The procedure for using InstaGene Matrix™ (BioRad, UK)

involve lysing the blood cells by addition of milliQ water (18.2 MΩ) and boiling the samples. Addition of the milliQ water also diluted out the PSG present in the stabilate, thus eliminated the inhibitory effect of PSG.

Boiling of the blood samples is necessary to lyse the parasite and denature the proteins such as haemoglobin, which inhibits PCR amplification reaction (Wuyts *et al.*, 1994). Boiling also removes anticoagulants (the stabilate contains heparin as anticoagulant), which has been reported as a potent PCR-amplification inhibitor (Newton and Graham, 1994).

The possibility of using stabilate for RAPD analysis has given greater opportunity to characterise more *T. evansi* stocks, which have been stored as stabilates in liquid nitrogen without employing the long steps for DNA purification. Wuyts *et al.* (1994), however, used blood samples which were allowed to clot and then boiled before DNA amplification for detecting *T. evansi* infection in livestock in Thailand.

Apart from complete lysing of the blood cells in the stabilate, this experiment also suggested the use of a fresh preparation of the stabilate DNA for the analysis to avoid DNA degradation after freezing and thawing.

The heterogeneity in the RAPD patterns of *T. evansi* stocks detected in this study had suggested that the RAPD analysis is a useful technique for stock characterisation in areas such as in Indonesia, where only one trypanosome species is present. Results from this study have confirmed the genetic heterogeneity among the *T. evansi* stocks in Indonesia, although the polymorphisms in the RAPD patterns were not as variable as detected by karyotyping using TAFE. Results from this study suggested that the RAPD analysis is useful for epidemiology studies in *T. evansi* stocks collected from widely distributed areas.

CHAPTER SIX

CHARACTERISATION OF *TRYPANOSOMA EVANSI* BY RIBOPRINTING

6.1. INTRODUCTION

Molecular techniques based on DNA and RNA sequence analysis for the characterisation of organisms for strain differentiation and phylogenetic studies developed rapidly. The analyses of the ribosomal RNA sequences have been shown to support the phylogenetic relationships more closely than DNA analysis (Kurtzman and Robnett, 1991). Initially ribosomal DNA sequencing techniques involved the gene cloning to provide sufficient rDNA for sequencing but the development of PCR-based techniques has replaced the need for lengthy cloning procedures. Ribosomal DNA fragment can now be amplified using oligonucleotide primers complementary to the targeted rDNA sequence. The sequencing technique, however, continues to be labour intensive and expensive.

Riboprinting techniques initially developed in *Entamoeba* by Clark and Diamond (1991a) involves polymerase chain reaction (PCR) amplification of the small subunit ribosomal RNA (SSU-rRNA) followed by digestion of the product with a range of restriction enzymes and separation of the resulting fragments by agarose gel electrophoresis. The primers used in riboprinting of trypanosomatids are conserved kinetoplastid SSU rDNA sequences which bind to the 5' and 3' end of the SSU-rRNA gene (Clark and Diamond, 1991a). The SSU rRNA genes are conserved in *Trypanosomatidae*, with sequence homologies gene observed in *T. cruzi*, *T. brucei* (Sogin *et al.*, 1986a); *L. donovani* (Looker *et al.*, 1988) and *C. fasciculata* (Schnare, Collings and Gray, 1986). Although the regions used for the primer are conserved, there are also regions of sequences between the primer sites that vary and the analysis of these is used in riboprinting. Hernandez *et al.* (1990) compared the SSU rRNA gene sequence of *T. cruzi* with the sequence of *T. brucei* (Sogin *et al.*, 1986a), *Leishmania donovani* (Looker *et al.*, 1988) and *Crithidia* (Schnare *et al.*, 1986) and assigned 8 universal and 9 variable regions in the SSU rRNA gene sequence. The presence of universal and variable regions in *Crithidia* had also been identified by Schnare *et al.* (1986).

Using riboprinting, Clark and Diamond (1991a) determined the taxonomic relationships among morphologically similar *Entamoeba* isolates and found that the "*Entamoeba histolytica*-like" amoebae are strains of *E. moskovskii* and not closely related to *E. histolytica*. The technique was used to distinguish between the 'pathogenic' and 'nonpathogenic' *E. histolytica* (Clark and Diamond, 1991b), which later were redescribed as two different species, *E. histolytica* (pathogenic) and *E. dispar* (nonpathogenic) (Diamond and Clark, 1993). Other applications of riboprinting including species identification (Clark & Diamond, 1991a; 1992; Brindley *et al.*, 1993; Brown & Jonckheere, 1994; Shen *et al.*, 1994; Clark *et al.*, 1995) and determination of taxonomic relationships (Molina *et al.*, 1992; Shen *et al.*, 1994; Brown and Jonckhere, 1994). Host specificity of rDNA variations in 17 *T. cruzi* isolates was demonstrated by Clark and Pung (1994) resulted in two distinct riboprint patterns on isolates cultured from opossum and raccoons, however, the pattern of trypanosome isolated from *Triatoma sanguisuga* was indistinguishable from those of the raccoon isolates. The analysis of the riboprint pattern may therefore be a useful indicator of insect vector preference for specific hosts (Clark, 1997a).

In the present study a riboprinting technique (Clark & Diamond, 1991a; Clark *et al.*, 1995) using the SSU-rDNA primers was used to examine stock differences of *Trypanosoma evansi* stocks from Indonesia.

6.2. MATERIALS AND METHODS

6.2.1. Trypanosomes

Trypanosomal DNA samples used in this study were obtained from three different DNA preparations: agarose embedded DNA, extracted DNA solution and DNA extracted directly from cryopreserved stabilate material. The trypanosome stocks used in the study are listed in **Table 6.1**.

Agarose embedded DNA of *T. evansi* stocks (7 stocks) representing the seven karyotype patterns identified previously in Chapter 4 ("Characterisation of *T. evansi* by pulsed-field gel electrophoresis"), Section 4.3.3. Additionally, *T. evansi* stocks from Brazil (TREU 2187) and Kenya (TREU 1810) were used in this study. Agarose embedded DNA of a *T. brucei* stock and a *T. congolense* stock were also included as outgroup species for reference purposes.

The DNA extracted directly from stabilate was prepared from 3 *T. evansi* stocks from Colombia (TREU 1179), Kenya (TREU 1733) and Sudan (TREU 1450) using the InstaGene® Matrix (BioRad, UK) described previously in Chapter 5 ("Characterisation of *T. evansi* stocks by random amplified polymorphic DNA analysis"), Section 5.5.2:

A *T. evansi* whole DNA preparation (Lun *et al.*, 1992b) of Chinese origin, a gift from Dr. Gibson, was also used for riboprint patterns comparison.

Table 6.1. Trypanosome stocks used for riboprinting.

No.	Stock No.	Isolation locality	Host
1	BAKIT 134/*T. evansi*	Ciawi, West Java	Buffalo
2	BAKIT 254/*T. evansi*	Minahasa, North Sulawesi	Horse
3	BAKIT 399/*T. evansi*	Tebingtinggi, North Sumatra	Buffalo
4	BAKIT 401/*T. evansi*	Tebingtinggi, North Sumatra	Buffalo
5	BAKIT 409/*T. evansi*	Menggala, Lampung	Cattle
6	BAKIT 427/*T. evansi*	Tebingtinggi, North Sumatra	Buffalo
7	BAKIT 461/*T. evansi*	Amuntai, South Kalimantan	Buffalo
8	BAKIT 467/*T. evansi*	Banyuwangi, East Java	Cattle
9	BAKIT 500/*T. evansi*	Bogor, West Java	Buffalo
10	BAKIT 508/*T. evansi*	Purworejo, Central Java	Buffalo
11	STIB 815/*T. evansi*	Guangdong, China	Horse
12	TREU 1179/*T. evansi*	Colombia	?
13	TREU 1450/*T. evansi*	Sudan	Camel
14	TREU 1733/*T. evansi*	Kenya	?
15	TREU 1810/*T. evansi*	Kenya	Camel
16	TREU 2177/*T. brucei*	Uganda	Cattle
17	TREU 2187/*T. evansi*	Brazil	Dog
18	TREU 2193/*T. congolense*	Nigeria	Cattle

6.2.2. Primers

The primers for riboprinting (Clark and Pung, 1994) derived from *T. cruzi* SSU-rDNA sequences (Hernandez *et al.*, 1990) which bind to the very 5' and 3' ends of the gene: KRD5: 5'-GATCTGGTTGATTCTGCCAGTAG-3' and KRD3: 5'-GATCCAGCTGCAGGTTCACCTAC were synthesised by Cruachem (UK).

6.2.3. PCR Amplification

The polymerase chain reaction conditions described by Clark and Diamond (1991a) was applied in this study. The PCR reaction mixtures contained: 10x PCR buffer (10 mM Tris-HCl pH 8.8, 25^0 C; 50 mM KCl; 1.5 mM $MgCl_2$; 0.1% TritonX-100); 0.2 μM each of the primers: KRD3 and KRD5; and 200 μM of dNTPs stock solution, and the DNA polymerase (DynaZyme, Finland) was added last at a concentration of 2 units per reaction. A drop of

mineral oil was added on top of the reaction mixture, to prevent evaporation during the amplification reaction. Approximately 10 ng of the *T. evansi* DNA contained in 1/8 of the agarose blocks was used as the PCR template.

The reaction mixtures were then pulse-centrifuged (3 seconds at 13,000 rpm) before placing them in the thermal cycler (OmniGene Thermal Cycler, Hybaid). The PCR conditions described by Clark and Diamond (1991a) were applied using Hybaid thermal cycler programmed for 30 cycles of 94^0 C for 1 min, 55^0 C for 1.5 min and 72^0 C for 2 min. The PCR product was detected on 5% polyacrylamide gel electrophoresis and visualised by silver staining as has been described in previous chapter (Chapter 5, Section 5.2.1.1.5).

6.2.4. Restriction Enzyme Analysis of the Amplification Products

PCR products were digested using the ten restriction enzymes that have been shown to detect inter-species differences among anuran trypanosomes (Clark *et al.*, 1995): *Hae*III, *Hha*I, *Hin*fI, *Rsa*I, *Msp*I, *Alu*I, *Taq*I, *Dde*I, *Sau*3AI, and *Scr*FI; all obtained, with their relevant buffer, from Boehringer Mannheim (**Table 6.2**). The PCR products amplified with the SSU-rRNA gene primers were stored in the refrigerator (4^0 C) and warmed at 50^0 C for 5 minutes in waterbath before used for the restriction digestion, to melt the agarose contained in the products. Warming up of the amplification products was also carried out with the PCR products that were not obtained from agarose blocks. Each of the amplification products was taken from the microfuge tubes in a 5 μl volume and used for the restriction enzyme digestion without prior purification. The amplification products were digested with a panel of 10 restriction enzymes for two hours at appropriate temperatures in a waterbath (**Table 6.2**). All reaction mixtures were identical in their component: 5 μl amplification product, 1 μl restriction enzyme buffer (10x), 10 units of restriction enzyme and type I deionised water (18.2 MΩ) added to make up a 10 μl volume.

Table 6.2. Restriction enzymes used for riboprinting study.

Enzyme	Recognition sequence	Enzyme activity (Units)	Optimal digestion temperature (^{0}C)
*Alu*I	5'-AG/CT-3'	10	37
*Dde*I	5'-C/TNAG-3'	10	37
*Hae*III	5'GG/CC-3'	10	37
*Hha*I	5'-GCG/C-3'	10	37
*Hin*fI	5'-G/ANTC-3'	10	37
*Msp*I	5'-C/CGG-3'	10	37
*Rsa*I	5'-GT/AC-3'	10	37
*Sau*3AI	5'-/GATC-3'	10	37
*Scr*FI	5'-CC/NGG-3'	10	37
*Taq*I	5'-T/CGA-3'	10	65

6.2.5. Gel Electrophoresis

Restriction enzyme digestion products were separated by electrophoresis in 5% polyacrylamide gel and the banding patterns were observed after the gel was stained by silver staining method described previously in Chapter 5. Each restriction enzyme digestion product was used as a 2.5 μl volume and mixed with 0.5 μl gel loading dye (Amersham, UK) before loading to the wells. Electrophoresis was carried out using BioRad Mini Protean II (BioRad, UK) system using 1 mm thick gel. The electrophoresis was performed at 200 V until the bromophenol blue reached the bottom of the gel (approximately 12-13 minutes). The band sizes were compared against a 1 kb ladder (Gibco, BRL) then photographed using Polaroid MP-4 Land Camera and filter no 45 (blue filter). The f-stop was set at 4.5 with 3-4 seconds exposure time and 45 seconds developing time. The banding pattern analysis was carried out according to the method described in Chapter 3, Section 3.3 (BioImage Analysis).

6.3. RESULTS

6.3.1. PCR Amplification with the SSU-rDNA Gene Primers

Each of the *T. evansi* stocks from Indonesia amplified with SSU-rDNA gene primers produced a band at approximately 2,200 bp (**Figure 6.1**). The amplification products in *T. evansi* stocks from Brazil (TREU 2187), Colombia (TREU 1179), Kenya (TREU 1810), Sudan (TREU 1450), *T. brucei* (TREU 2177) and *T. congolense* (TREU 2193) with the SSU-rDNA primers are presented in **Figure 6.2**. Each stock produced a band at approximately 2,200 bp except TREU 1179 (lane 3, **Figure 6.2**) and TREU 1450 (lane 5, **Figure 6.2**) which did not show any bands after PCR amplification.

Figure 6.1. Amplification product of *Trypanosoma evansi* stocks with the small subunit ribosomal RNA gene primers detected in 5% silver stained polyacrylamide gel showing a fragment of 2,200 bp length.

Track 1: 1 kb standard DNA size marker
Track 2: BAKIT 134
Track 3: BAKIT 508
Track 4: BAKIT 401
Track 5: BAKIT 467
Track 6: BAKIT 461
Track 7: BAKIT 500
Track 8: BAKIT 427
Track 9: STIB 815

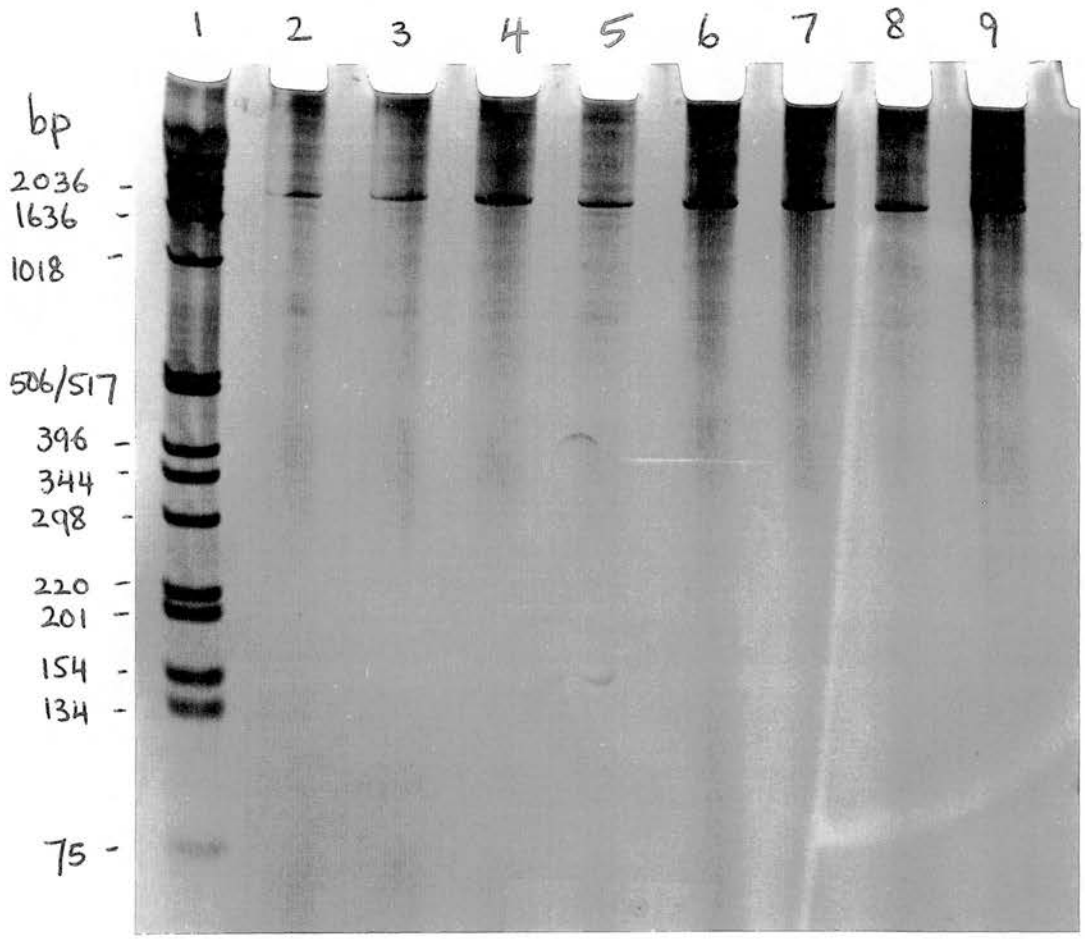
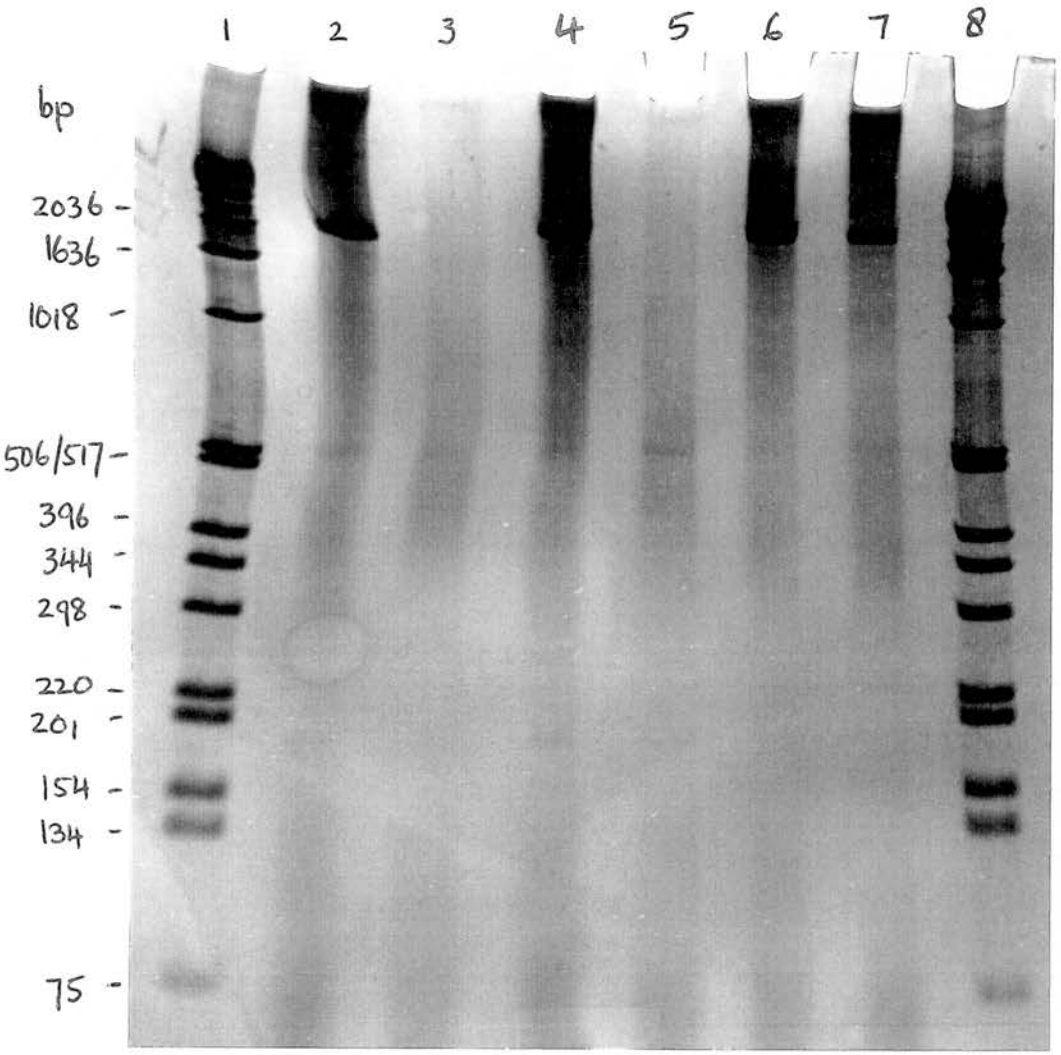

1
2
3
4
5
6
7
8
9
bp
2036
1636
1018
506/517
396
344
298
220
201
154
134
75

Figure 6.2. Amplification product of trypanosome stocks with the small subunit ribosomal RNA gene detected in 5% silver stained polyacrylamide gel showing a fragment of 2,200 bp length.

Tracks1, 8: 1 kb DNA standard size marker,
Track 2: TREU 2187
Track 3: TREU 1179
Track 4: TREU 1810
Track 5: TREU 1450
Track 6: TREU 2177
Track 7: TREU 2193


1
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bp
2036
1636
1018
506/517
396
344
298
220
201
154
134
75

6.3.2. Riboprinting

Riboprinting of the Indonesian *T. evansi* (7), *T. brucei* (1) and *T. congolense* (1) stocks showed distinct inter-species differences in the banding patterns in the 148-1,018 bp size range after digestion with *Scr*FI (**Figure 6.3**). However, riboprint patterns variations were not observed among the *T. evansi* stocks from Indonesia which all produced six bands between 148-581 bp size range according to the enzyme used. The band at 235 bp, however, was fainter in some stocks (lane 2, 4 and 6, **Figure 6.3**). The riboprint pattern of the *T. brucei* stock differed from the Indonesian *T. evansi* in the absence of the band at 235 bp in *T. brucei* but the riboprint pattern of the Kenyan *T. evansi* was identical to that of *T. brucei*. The *T. congolense* stock tested showed completely different riboprint pattern to those of *T. evansi* and *T. brucei* with five bands between 200-1,018 bp size range in *T. congolense*. Common bands shared between *Trypanozoon* (*T. brucei* and *T. evansi*) and *Nannomonas* (*T. congolense)* studied were not observed.

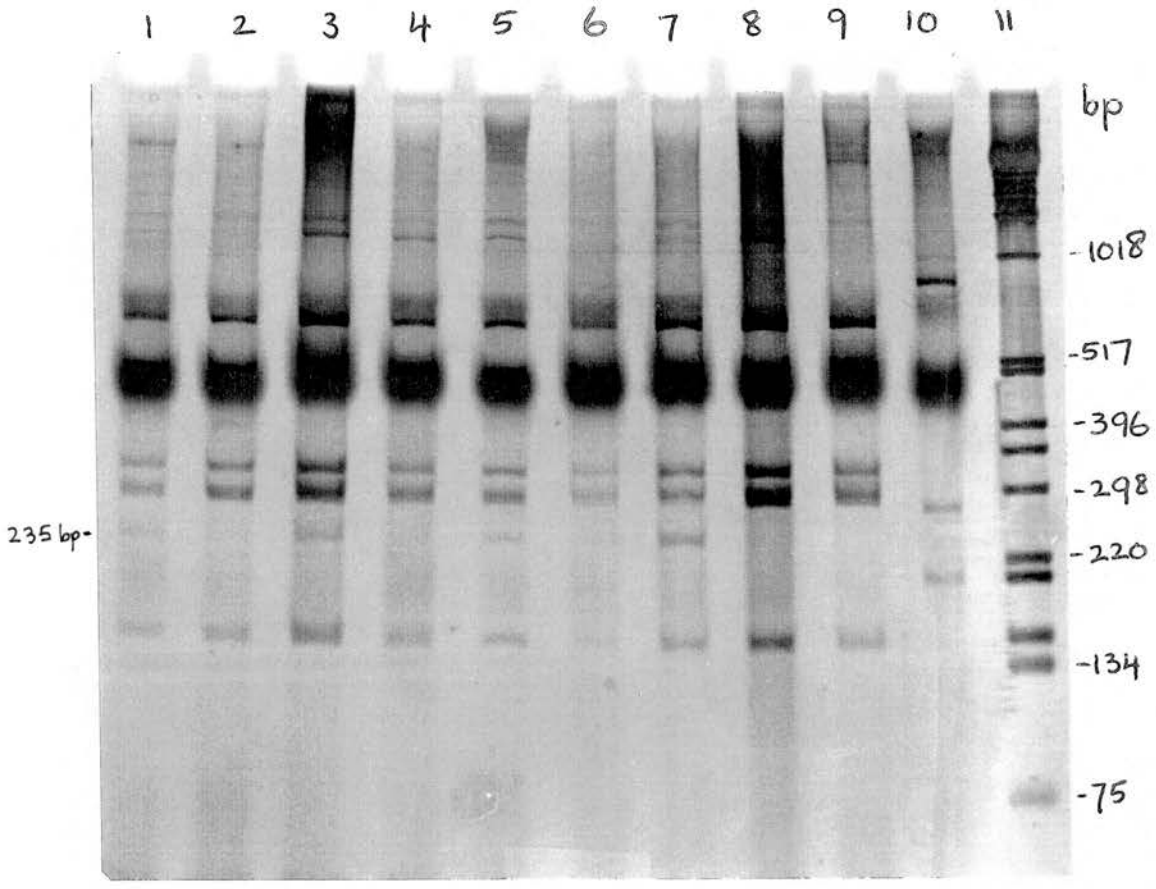
Subgeneric differences in the riboprint patterns were also observed after digestion with *Hha*I (**Figure 6.4**); *Hae*III (**Figure 6.5**); *Taq*I (**Figure 6.6**); *Msp*I (**Figure 6.7**); *Hinf*I (**Figure 6.8**) and *Dde*I (**Figure 6.9**). The banding patterns shown by post-restriction digested products with those enzymes (*Hha*I, *Hae*III, *Taq*I, *Msp*I, *Hinf*I, and *Dde*I) of *T. evansi* were similar to that of *T. brucei* but differed from *T. congolense* (**Figure 6.4** to **6.9**).

Seven bands between 87-605 bp size range with identical banding patterns were seen in the restriction pattern of *T. evansi* and *T. brucei* after digestion with *Hha*I (**Figure 6.4**). The riboprint pattern of *T. congolense* was different from those of *T. evansi* and *T. brucei* with 8 bands in the size range of 87-500 bp detected in *T. congolense*. Two common bands (87 and 188 bp), however, were shared among the restriction patterns of *T. evansi*, *T. brucei* and *T. congolense*.

Riboprint patterns of the amplification product digested with *Hae*III (**Figure 6.5**) produced 8 bands in the size range between 148-731 bp in *T. evansi* and *T. brucei*. The riboprint pattern of *T. congolense* was different from those of *T. evansi* and *T. brucei* with 6 bands between 120-650 bp size range seen in *T. congolense*. Although the riboprint patterns of *T. evansi* and *T. brucei* were different from *T. congolense*, two bands at 611 bp and 536 bp were common in all stocks tested.

Figure 6.3. Riboprint patterns of *Trypanosoma brucei*, *Trypanosoma congolense* and *Trypanosoma evansi* stocks from Indonesia and Kenya obtained after endonuclease digestion with *Scr*FI.

Track11: 1 kb standard DNA size marker
Track 1: BAKIT 134
Track 2: BAKIT 508
Track 3: BAKIT 401
Track 4: BAKIT 467
Track 5: BAKIT 461
Track 6: BAKIT 500
Track 7: BAKIT 427
Track 8: TREU 1810
Track 9: TREU 2177
Track10: TREU 2193


1
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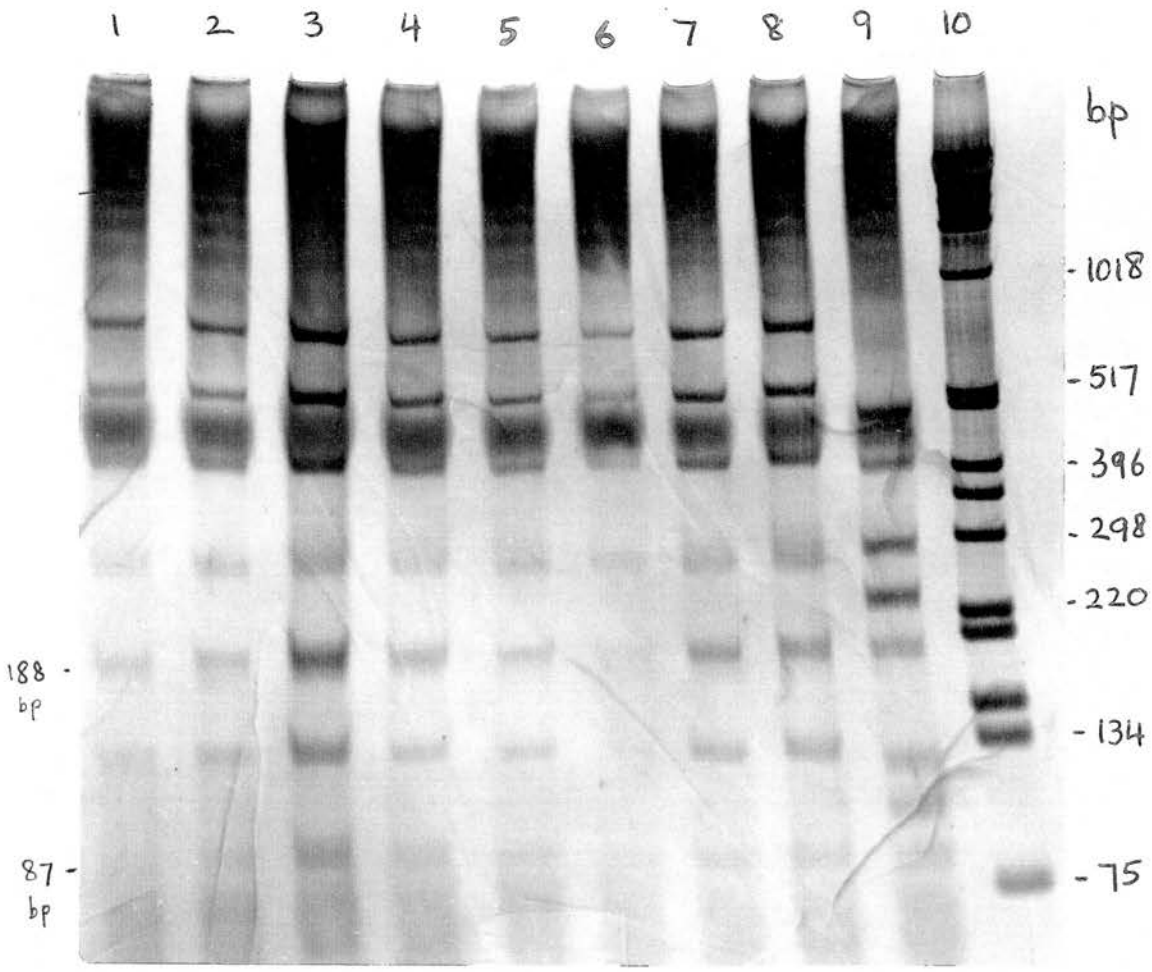
6
7
8
9
10
11
bp
1018
517
396
298
235 bp
220
134
75

Figure 6.4. Riboprint patterns of *Trypanosoma brucei*, *Trypanosoma congolense* and *Trypanosoma evansi* stocks obtained after endonuclease digestion with *Hha*I.

Track 10: 1 kb standard DNA size marker
Track 1: BAKIT 134
Track 2: BAKIT 508
Track 3: BAKIT 401
Track 4: BAKIT 467
Track 5: BAKIT 461
Track 6: BAKIT 500
Track 7: BAKIT 427
Track 8: TREU 2177
Track 9: TREU 2193


1
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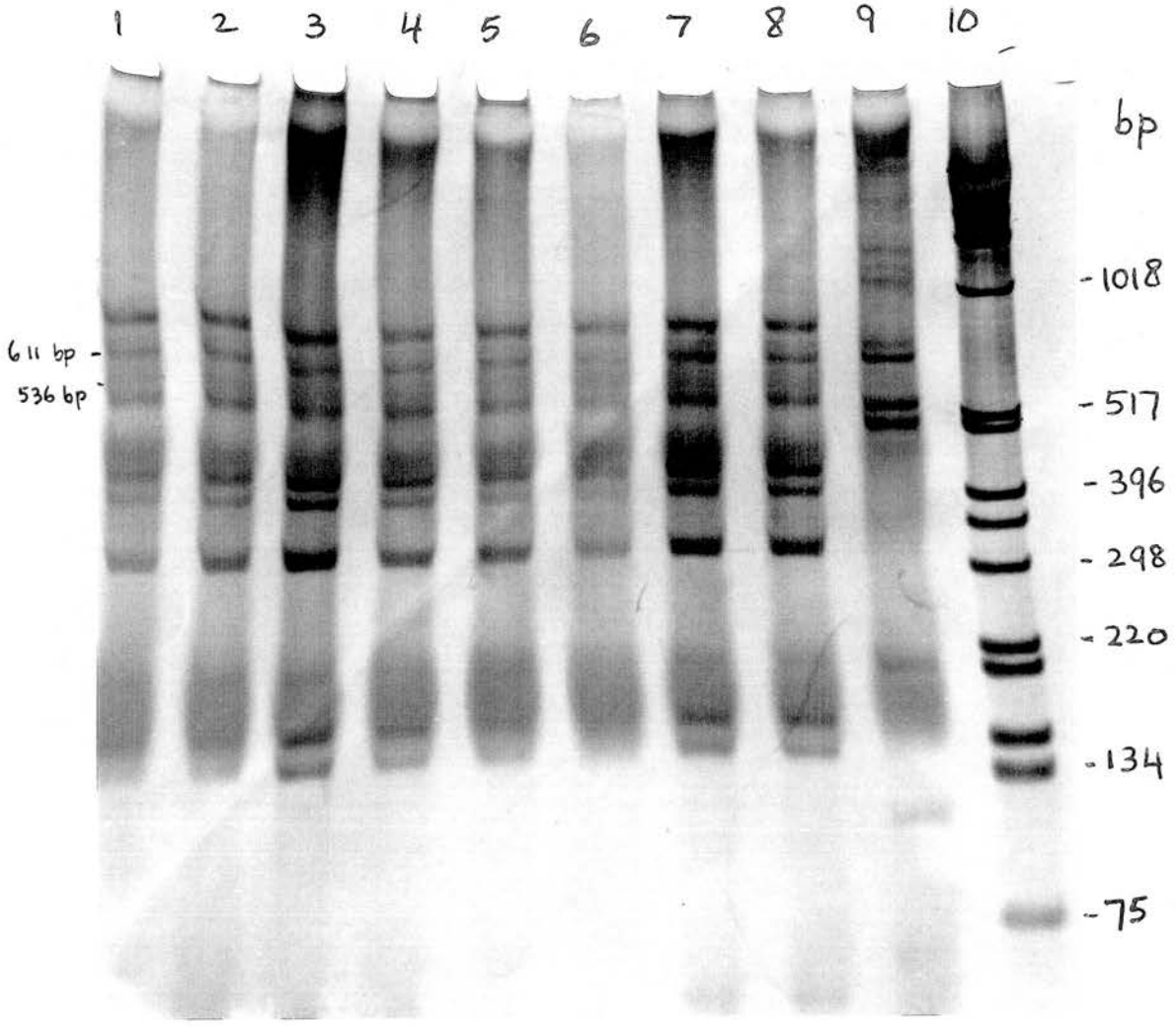
3
4
5
6
7
8
9
10
bp
1018
517
396
298
220
188 bp
134
87 bp
75

Figure 6.5. Riboprint patterns of *Trypanosoma brucei*, *Trypanosoma congolense* and *Trypanosoma evansi* stocks obtained after endonuclease digestion with *Hae*III.

Track 10: 1 kb standard DNA size marker
Track 1: BAKIT 134
Track 2: BAKIT 508
Track 3: BAKIT 401
Track 4: BAKIT 467
Track 5: BAKIT 461
Track 6: BAKIT 500
Track 7: BAKIT 427
Track 8: TREU 2177
Track 9: TREU 2193


1
2
3
4
5
6
7
8
9
10
bp
611 bp
536 bp
1018
517
396
298
220
134
75

Identical riboprint patterns were seen in the restriction digestion products with *Taq*I in *T. evansi* and *T. brucei* stocks with the presence of 6 bands between 72-1,018 bp size range (**Figure 6.6**). *T. congolense* showed different riboprint pattern from those of *T. evansi* and *T. brucei* with the presence of 7 bands in the size range of 72-760 bp in *T. congolense*, however, 3 bands were common in all stocks tested: 441 bp, 86 bp and 72 bp.

Restriction enzyme digestion of the amplification products with *Msp*I (**Figure 6.7**) showed identical banding patterns with 6 bands in the size range of 138-906 bp in *T. evansi* and *T. brucei* stocks. Six bands in the size range between 192-891 bp were detected in *T. congolense* with different banding pattern from those of *T. evansi* and *T. brucei*. Three bands were common in all of the trypanosome stocks tested: 906 bp, 490 bp and 301 bp.

The riboprint patterns shown by trypanosome stock after digestion with restriction enzyme *Hinf*I was presented in **Figure 6.8**. The riboprint patterns of *T. evansi* and *T. brucei* stocks were identical and different from that of *T. congolense*. Seven bands in the size range of 101-1081 bp in *T. evansi* and *T. brucei* stocks and 7 bands between 78-831 bp in *T. congolense* were observed. Two bands at 259 bp and 135 bp were common in all stocks tested.

Riboprint patterns in trypanosome stocks after digested with *Dde*I are shown in **Figure 6.9**. Six bands in the size range of 162-652 bp were observed in *T. evansi* and *T. brucei* stocks and 3 bands were seen in the size range of 281-652 bp in *T. congolense*. Two bands were common in the trypanosome stocks tested: 289 bp and 652 bp.

Identical riboprint pattern in *T. evansi*, *T. brucei* and *T. congolense* stocks studied was observed in the amplification products after digested with *Alu*I (**Figure 6.10**). Seven bands were observed between 155-650 bp size range.

All *T. evansi* stocks revealed identical riboprint patterns after digestion with *Rsa*I with 6 bands observed between 145-1400 bp size range (**Figure 6.11**). The banding pattern shown by the restriction products with *Rsa*I of *T. evansi* was different from those of *T. brucei* and *T. congolense*. Riboprint pattern of *T. brucei* (lane 8 in **Figure 6.11**) was identical to that of *T. congolense* (lane 9 in **Figure 6.11**).

The riboprint pattern of *T. congolense* stock was similar to those of the *T. evansi* stocks tested after digested with *Sau*3AI (**Figure 6.12**). Six bands in the size range of 175-1,100 bp were detected. Three stocks (tracks 3, 6 and 8 in **Figure 6.12**) did not show clear banding pattern, these three stocks were excluded in the analysis.

Figure 6.6. Riboprint patterns of *Trypanosoma brucei*, *Trypanosoma congolense* and *Trypanosoma evansi* stocks obtained after endonuclease digestion with *Taq*I.

Track10: 1 kb standard DNA size marker
Track 1: BAKIT 134
Track 2: BAKIT 508
Track 3: BAKIT 401
Track 4: BAKIT 467
Track 5: BAKIT 461
Track 6: BAKIT 500
Track 7: BAKIT 427
Track 8: TREU 2177
Track 9: TREU 2193

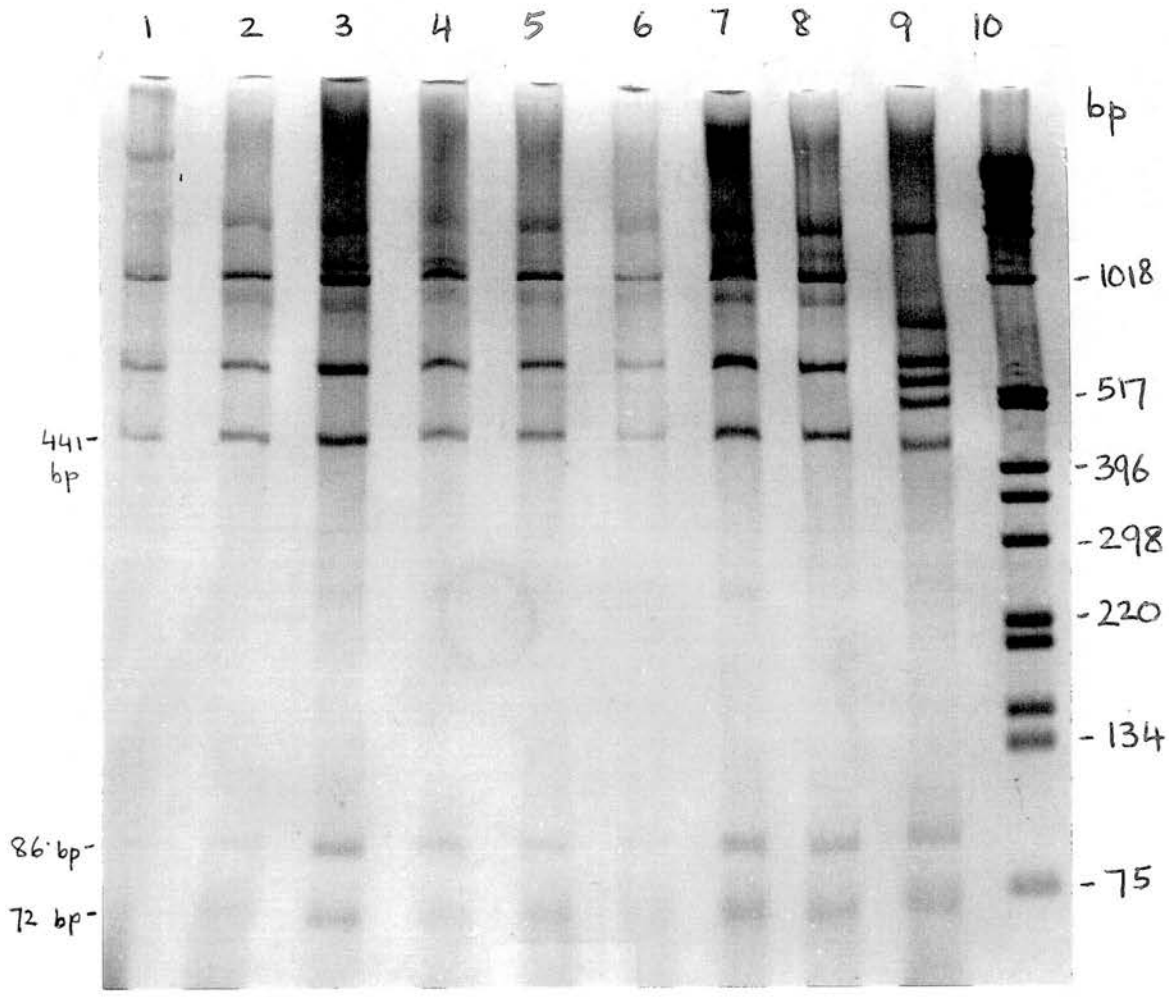
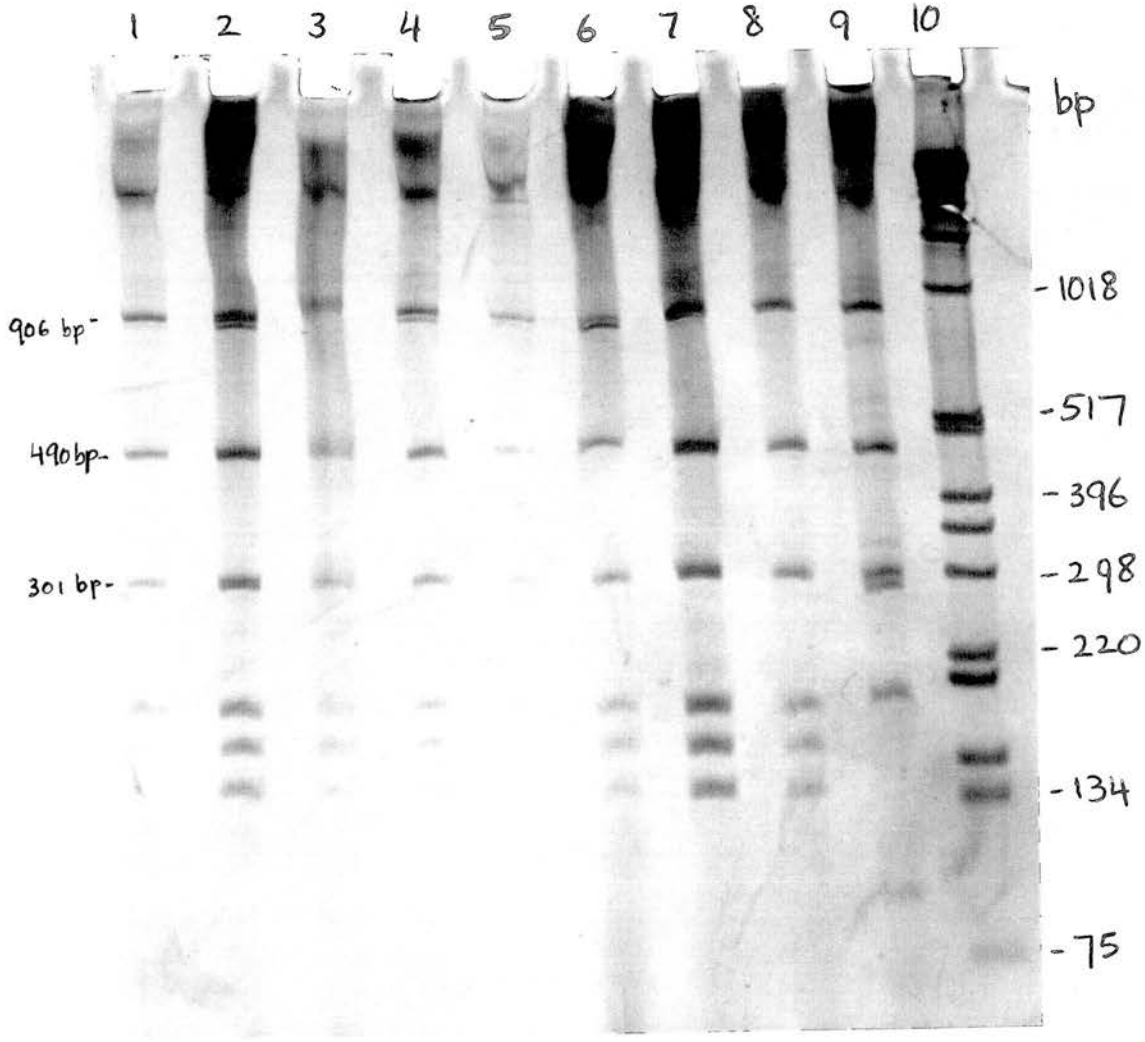

1
2
3
4
5
6
7
8
9
10
bp
1018
517
441-
bp
396
298
220
134
86 bp
72 bp
75

Figure 6.7. Riboprint patterns of *Trypanosoma brucei*, *Trypanosoma congolense* and *Trypanosoma evansi* stocks after endonuclease digestion with *Msp*I.

Track10: 1 kb standard DNA size marker
Track 1: BAKIT 134
Track 2: BAKIT 508
Track 3: BAKIT 401
Track 4: BAKIT 467
Track 5: BAKIT 461
Track 6: BAKIT 500
Track 7: BAKIT 427
Track 8: TREU 2177
Track 9: TREU 2193


1
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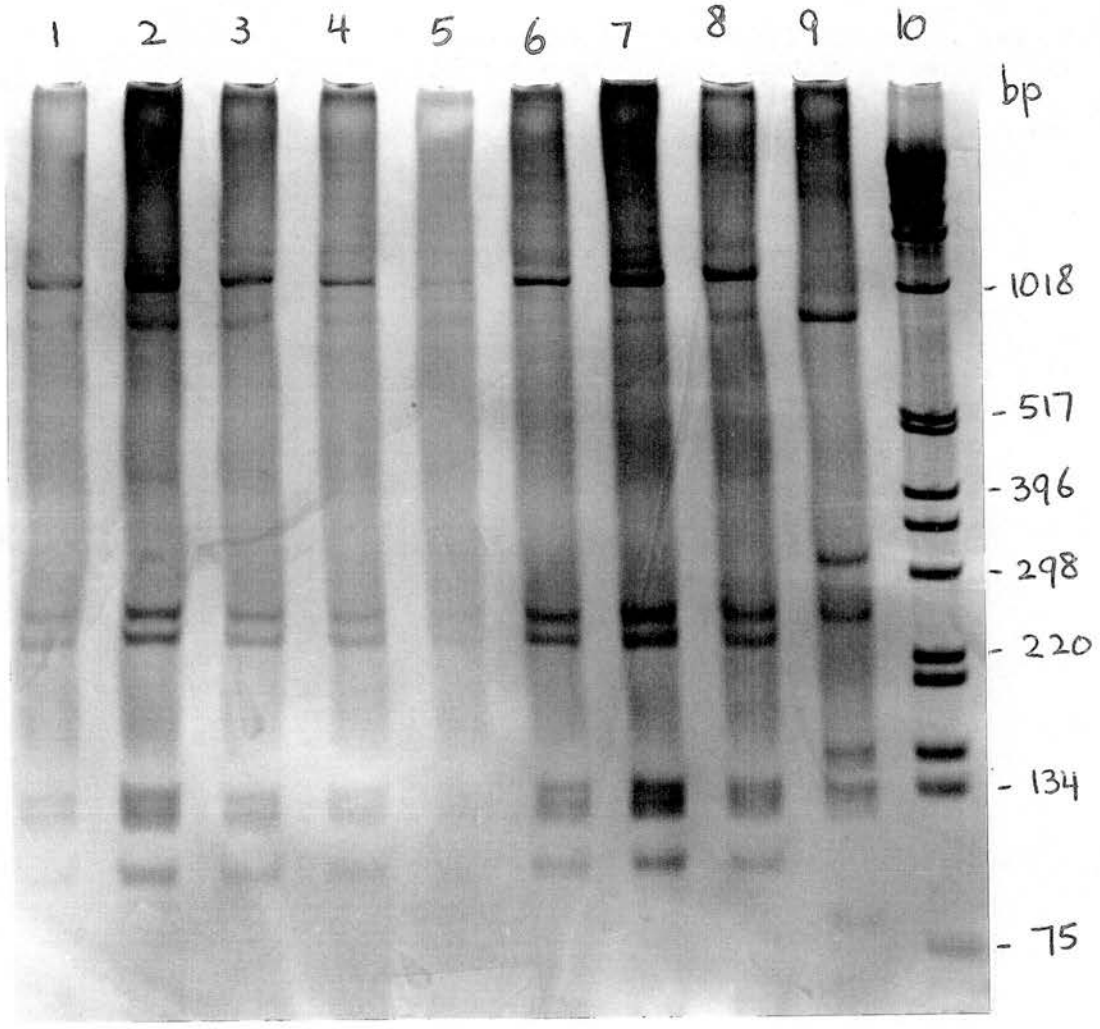
7
8
9
10
bp
906 bp
490bp
301 bp
1018
517
396
298
220
134
75

Figure 6.8. Riboprint patterns of *Trypanosoma brucei*, *Trypanosoma congolense* and *Trypanosoma evansi* stocks obtained after endonuclease digestion with *Hinf*I.

Track10: 1 kb standard DNA size marker
Track 1: BAKIT 134
Track 2: BAKIT 508
Track 3: BAKIT 401
Track 4: BAKIT 467
Track 5: BAKIT 461
Track 6: BAKIT 500
Track 7: BAKIT 427
Track 8: TREU 2177
Track 9: TREU 2193


1
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9
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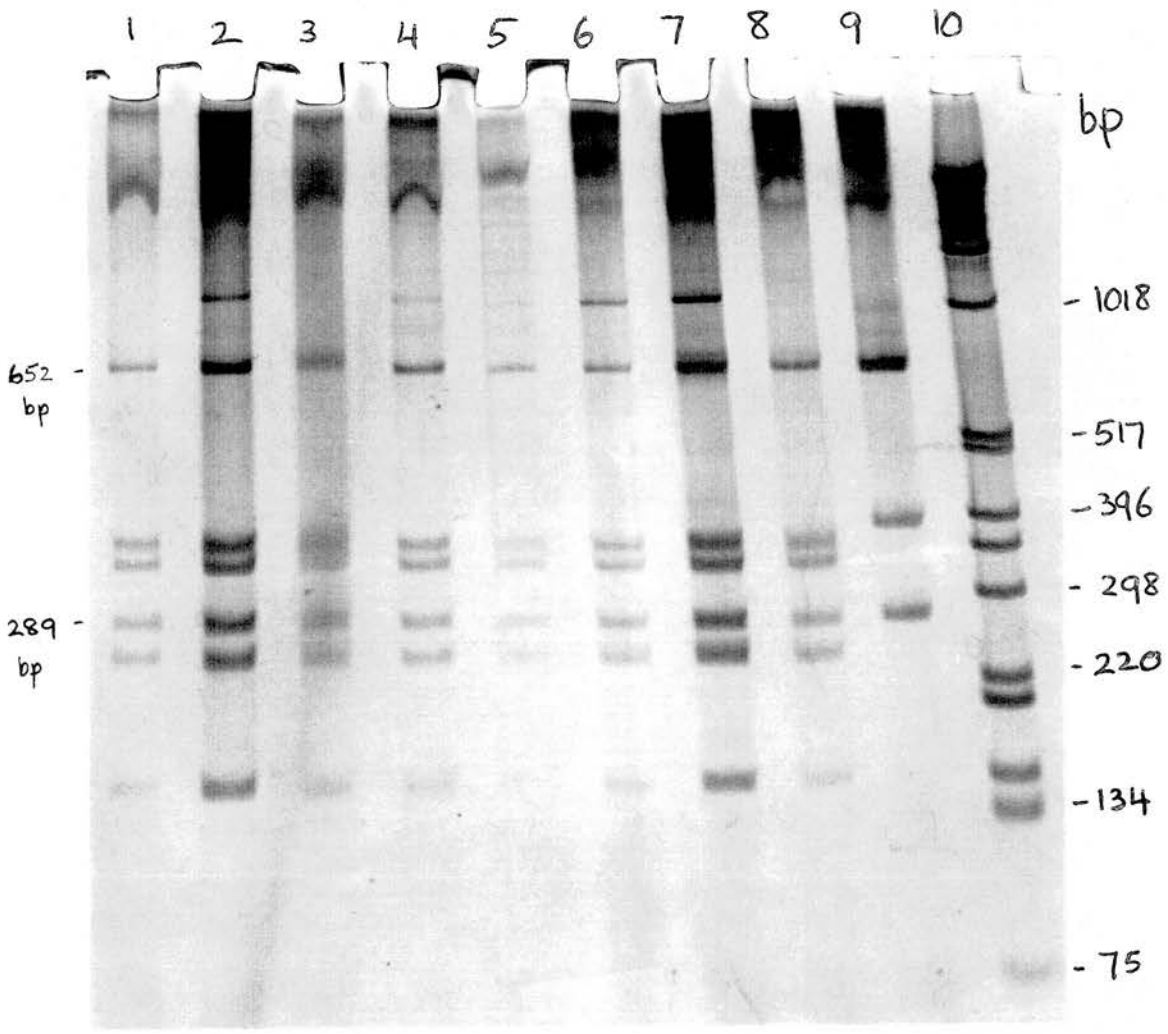
bp
1018
517
396
298
220
134
75

Figure 6.9. Riboprint patterns of *Trypanosoma brucei*, *Trypanosoma congolense* and *Trypanosoma evansi* stocks obtained after endonuclease digestion with *Dde*I.

Track10: 1 kb standard DNA size marker
Track 1: BAKIT 134
Track 2: BAKIT 508
Track 3: BAKIT 401
Track 4: BAKIT 467
Track 5: BAKIT 461
Track 6: BAKIT 500
Track 7: BAKIT 427
Track 8: TREU 2177
Track 9: TREU 2193


1
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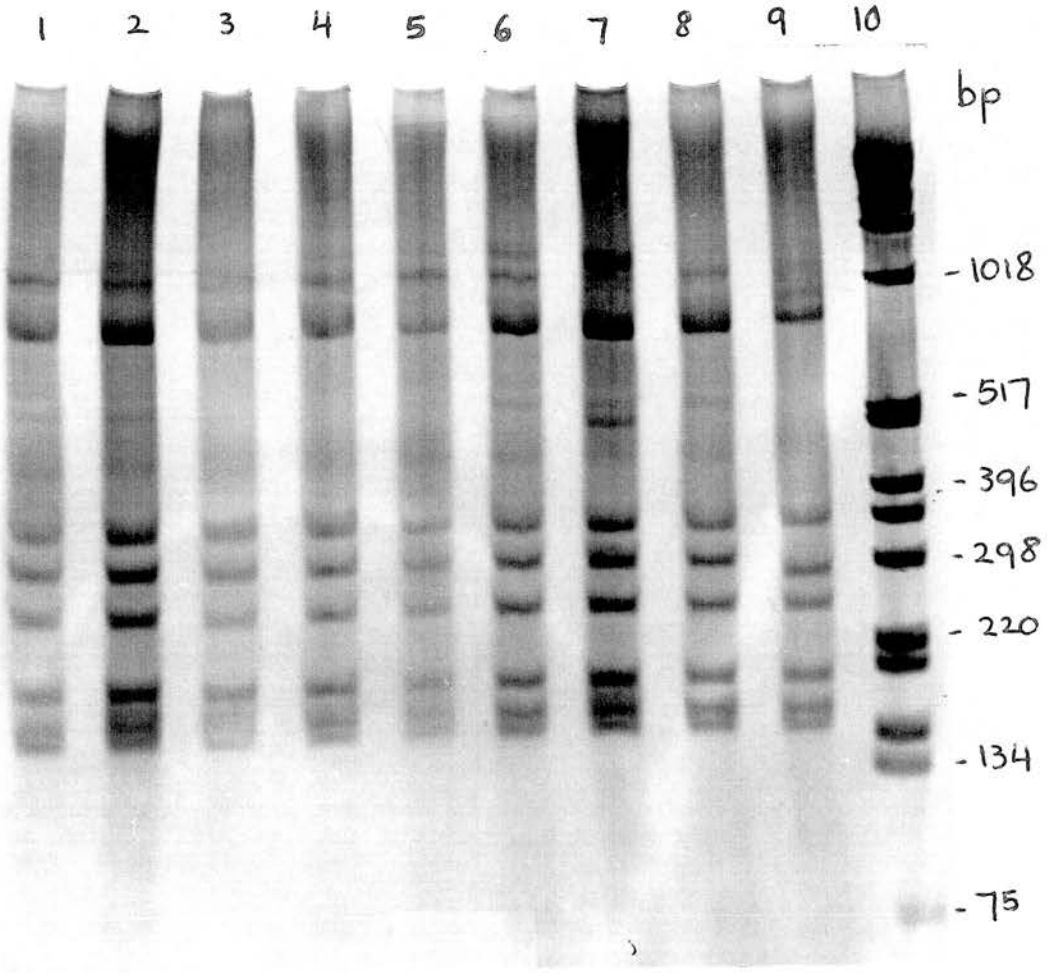
7
8
9
10
bp
1018
652 bp
517
396
298
289 bp
220
134
75

Figure 6.10. Riboprint patterns of *Trypanosoma brucei*, *Trypanosoma congolense* and *Trypanosoma evansi* stocks obtained after endonuclease digestion with *Alu*I.

Track10: 1 kb standard DNA size marker
Track 1: BAKIT 134
Track 2: BAKIT 508
Track 3: BAKIT 401
Track 4: BAKIT 467
Track 5: BAKIT 461
Track 6: BAKIT 500
Track 7: BAKIT 427
Track 8: TREU 2177
Track 9: TREU 2193


1
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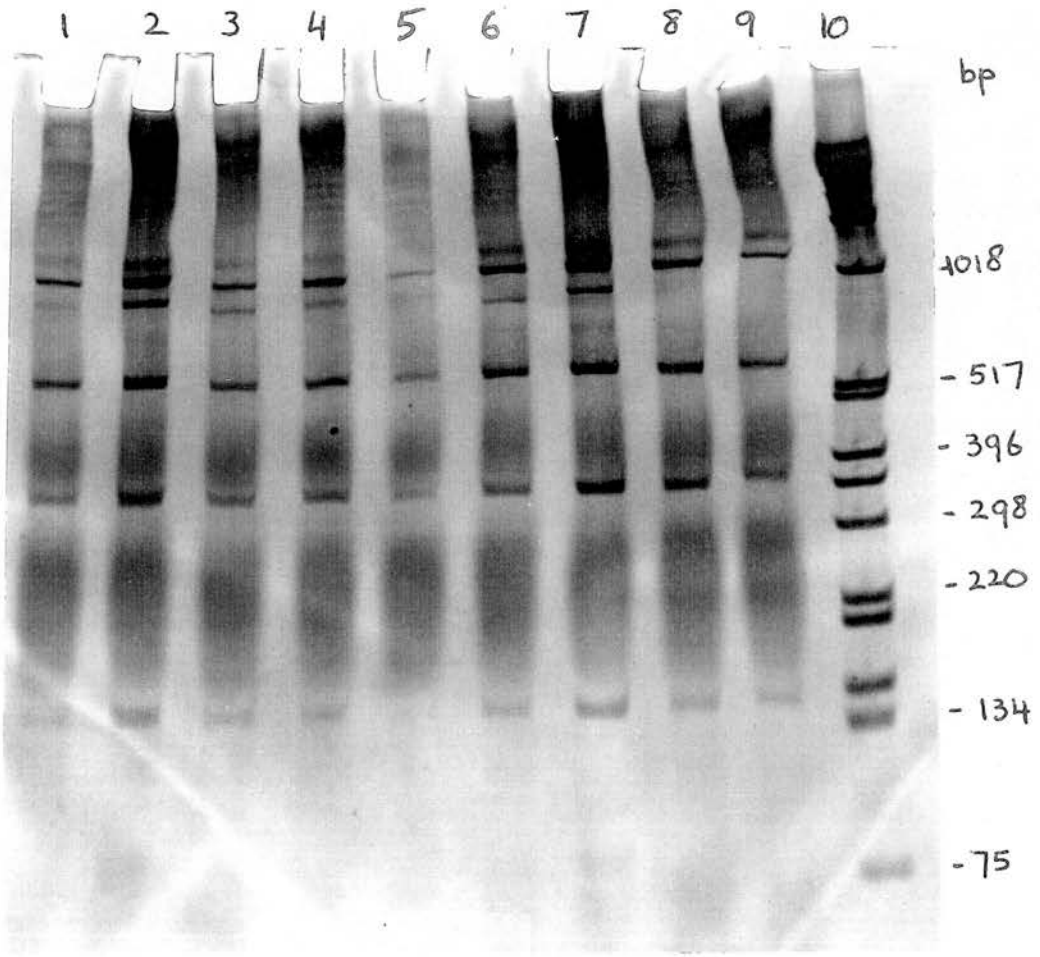
9
10
bp
1018
517
396
298
220
134
75

Figure 6.11. Riboprint patterns of *Trypanosoma brucei*, *Trypanosoma congolense* and *Trypanosoma evansi* stocks obtained after endonuclease digestion with *Rsa*I.

Track10: 1 kb standard DNA size marker
Track 1: BAKIT 134
Track 2: BAKIT 508
Track 3: BAKIT 401
Track 4: BAKIT 467
Track 5: BAKIT 461
Track 6: BAKIT 500
Track 7: BAKIT 427
Track 8: TREU 2177
Track 9: TREU 2193


1
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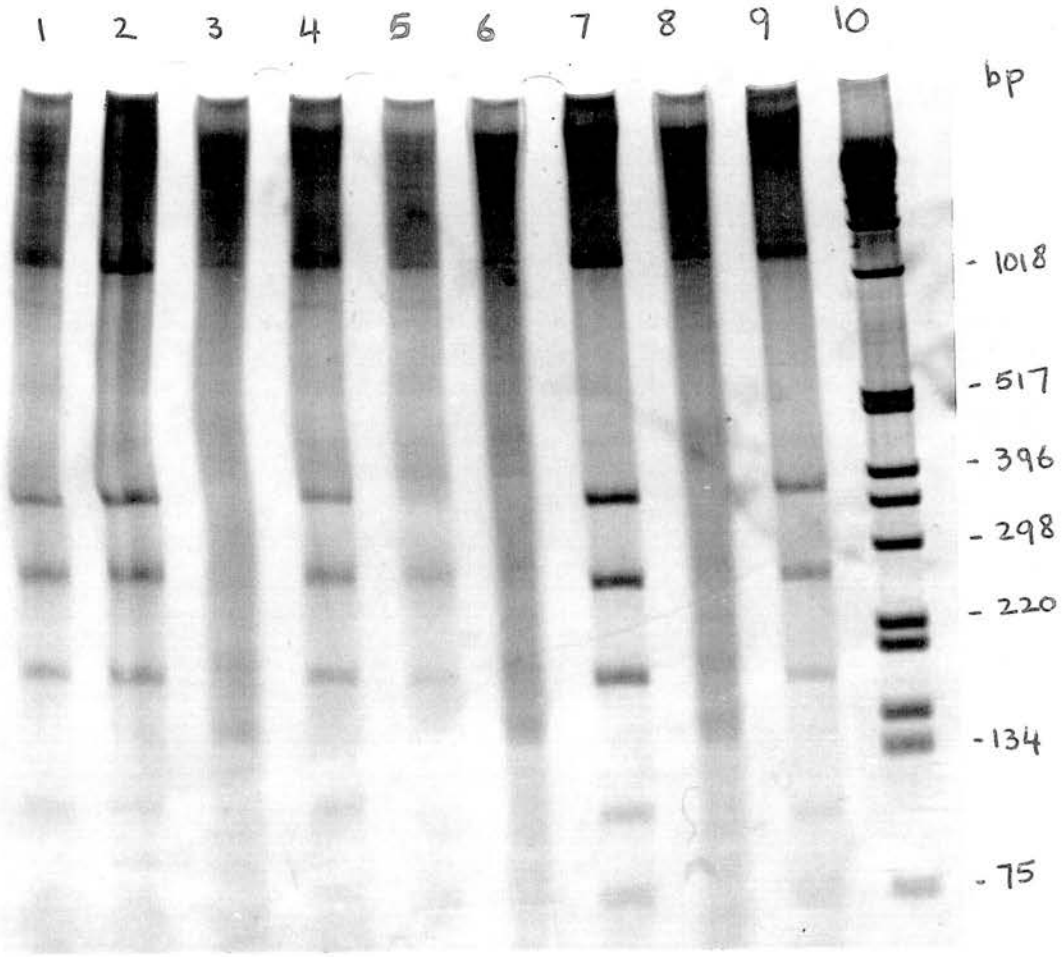
7
8
9
10
bp
1018
517
396
298
220
134
75

Figure 6.12. Riboprint patterns of *Trypanosoma brucei*, *Trypanosoma congolense* and *Trypanosoma evansi* stocks obtained after endonuclease digestion with *Sau*3AI.

Track10: 1 kb standard DNA size marker
Track 1: BAKIT 134
Track 2: BAKIT 508
Track 3: BAKIT 401
Track 4: BAKIT 467
Track 5: BAKIT 461
Track 6: BAKIT 500
Track 7: BAKIT 427
Track 8: TREU 2177
Track 9: TREU 2193


1
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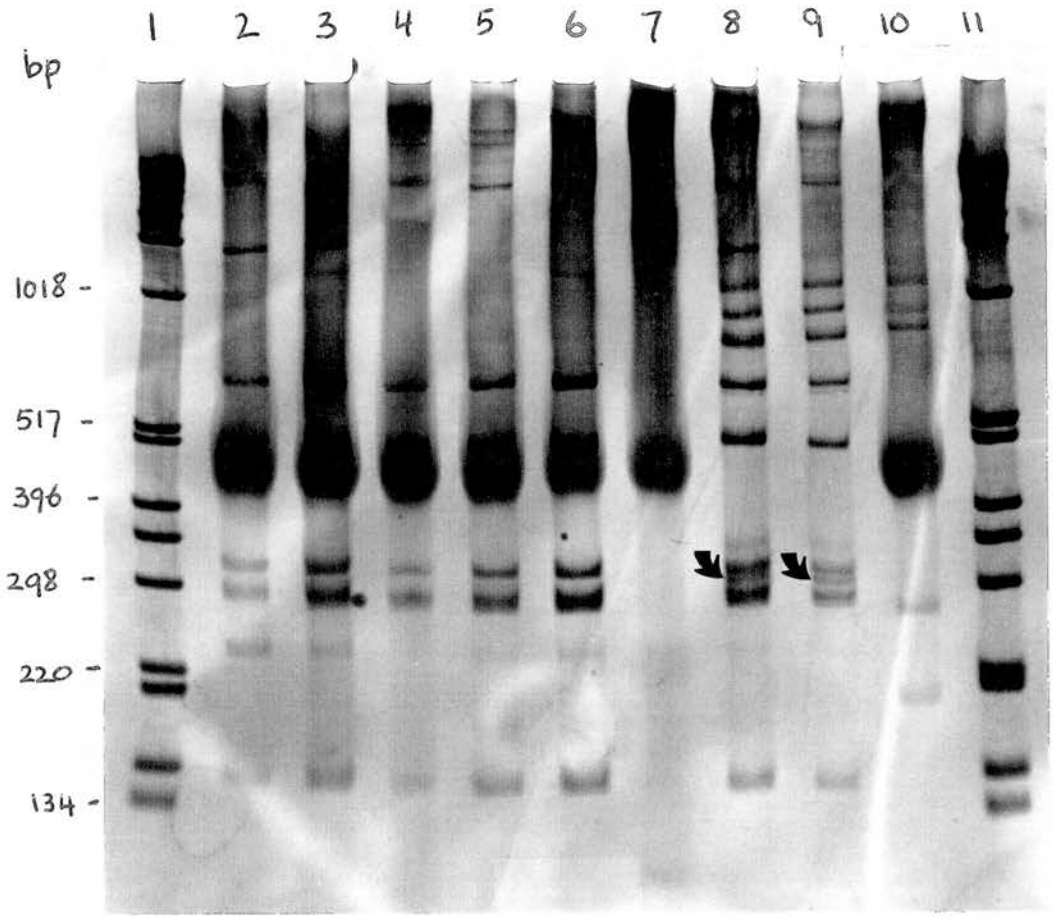
9
10
bp
1018
517
396
298
220
134
75

Figure 6.13. Riboprint patterns of *Trypanosoma evansi*, *Trypanosoma brucei* and *Trypanosoma congolense* stocks obtained after digestion with *Scr*FI.

Tracks1, 11: 1 kb DNA standard size marker
Track 2: BAKIT 409 (Indonesia)
Track 3: BAKIT 399 (Indonesia)
Track 4: BAKIT 254 (Indonesia)
Track 5: STIB 815 (China)
Track 6: TREU 2187 (Brazil)
Track 7: TREU 1733 (Kenya)
Track 8: TREU 1810 (Kenya)
Track 9: TREU 2177
Track10: TREU 2193



↘ 300 bp

A further study carried out to examine potential intra-species differences in riboprinting patterns in *T. evansi* stocks isolated from Kenya, Brazil, China and 3 other Indonesian stocks after digestion with *Scr*FI (**Figure 6.13**). It was seen that the riboprint patterns shown by *T. evansi* stocks from Indonesia (lane 2-4, **Figure 6.13**), China (lane 5, **Figure 6.13**) and Brazil (lane 6, **Figure 6.13**) were similar. One of *T. evansi* stocks from Kenya, TREU 1733 (Lane 7, **Figure 6.13**), however did not show any banding pattern after digestion, the DNA having been prepared directly from stabilate using InstaGene® Matrix (BioRad, UK). The riboprint pattern of the other *T. evansi* stock from Kenya (TREU 1810) was identical to that of *T. brucei* (**Figure 6.13**).

6.4. DISCUSSION

The PCR amplification of SSU-rRNA of each of the trypanosome stocks yielded a product with fragment length of approximately 2,200 bp, which is similar to that reported by Clark *et al.* (1995) in trypanosome stocks isolated from toads and frogs. Clark (1997a) observed that most of the eukaryotes amplified with SSU-rDNA generated products between 1,700-2,200 bp in length. Evidence of SSU-rRNA gene variation was also detected by restriction enzyme digestion as reported by Clark and Diamond (1991a).

The present study showed that DNA samples prepared directly from cryostabilates did not produce any bands after PCR amplification (lane 3 and 5 in **Figure 6.2**) or after enzyme digestion (lane 7 in **Figure 6.13**). This might be caused by degradation of intact DNA due to the nuclease activity. Moreover, because of the large DNA involved, the trypanosome DNA is likely to shear during the process of the sample preparation using InstaGene ® matrix (BioRad, UK) which involves centrifugation and vortexing at high speed. DNA samples embedded in agarose, however, are protected against shearing by the agarose during the sample preparation.

Polymorphisms in the riboprint patterns were only detected in the products digested with *Scr*FI; which produced distinct patterns for *T. evansi*, *T. brucei* and *T. congolense* indicating the presence of sequence variation in the subgenus level of *Trypanozoon*. However, riboprint patterns of species other than *T. evansi* and *T. brucei* in the genus *Trypanozoon,* were not tested in the present study. The results of the present study were in agreement with those of Clark *et al.* (1995) who reported that intra-specific variations in riboprint patterns were not found in the 20 trypanosome isolates from Anuran vertebrates.

Riboprinting has been suggested as a useful diagnostic tool for species identification for other protozoa *Entamoeba gingivalis* (Clark and Diamond, 1992) and *Toxoplasma gondii* (Brindley *et al.*, 1993) from their closely related species. However, it can be concluded that riboprinting is a useful tool only for species differentiation in areas where there is more than

one clearly separated species of pathogen present. In areas such as in Africa or in South America, where more than one trypanosome species is present, riboprinting can be used as an aid of accurate diagnosis of the species involved in the infection. Accuracy in the diagnosis of the species involved is important in the application of specific therapeutic intervention to control the disease in such areas.

Results from this study have shown the riboprint patterns differentiate stocks from Kenya and stocks from Indonesia, China and Brazil (**Figure 6.13**). The term ribodeme was proposed by Clark and Pung (1994) to describe populations of a species, which have indistinguishable riboprint pattern. These results might suggest that the Indonesian *T. evansi* did not originate directly from Africa.

A close similarity in the riboprint patterns was observed between the Kenyan *T. evansi* and *T. brucei* stocks after digestion with *Scr*FI (**Figure 6.3**). The study also showed that the amplification products of *T. evansi* and *T. brucei* after digestion with *Alu*I showed identical riboprint pattern to that of *T. congolense* (**Figure 6.10**). Clark (1997a) suggested however that identical riboprints do not indicate that the organisms belong to the same species because it is possible for different species to have identical SSU-rRNA sequence (Sogin, Ingold, Karlok, *et al.*, 1986b).

This study has shown that restriction fragment length polymorphisms of SSU rDNA of *T. evansi* and *T. brucei* digested with six different enzymes (*Hha*I, *Hae*III, *Taq*I, *Msp*I, *Hinf*I and *Dde*I) revealed similar restriction patterns but differed from that of *T. congolense* (**Figure 6.4** to **6.9**). This has also shown that riboprinting provides evidence for the accepted closer relationships of *T. evansi* to *T. brucei* than to *T. congolense* (Hoare, 1972).

Intra-specific variations in riboprints were not detected in this study. In general, intra-specific variations in riboprint patterns are generated by the presence of one or more extra restriction sites within the SSU-rRNA gene sequence (Vaneechoutte *et al.*, 1992) and occur in the variable regions of rRNA genes. An intra-specific variation in the riboprint patterns is unusual (Clark and Pung, 1994). However, strain differences have been reported (Clark and Pung, 1994) in 17 sylvatic *T. cruzi* isolates from mammals revealing 3 distinct patterns (3 ribodemes) that correlated to the host from which they had been isolated (raccoon, opossum and human). Strain differences have also been found in *Entamoeba* in 8 isolates of *Entamoeba moshkovskii* have 5 distinct riboprint patterns and the 5 "*E. histolytica-like*" strains tested had identical riboprint pattern to one of *E. moshkovskii* (Clark and Diamond, 1991a). Intra-specific differences were also reported in *Blastocystis hominis*, a common human parasite of unknown role in human disease; however, the significance of the differences of riboprint patterns in this parasite is still unknown (Clark, 1997b).

One of the disadvantages of riboprinting is that different electrophoretic patterns can be generated by the presence of a single extra restriction site (Vaneechoutte *et al.*, 1992). Riboprinting only detects a small proportion of the gene sequence (Brown and De Jonckheere, 1994) and significant variation may remain undetected unless that variation corresponds to a restriction site (Clark, 1997a). It was further suggested (Clark, 1997a) that riboprint alone should not be used as the basis for new species determination. Other techniques, such as genetic, immunological and biochemical data, should also be used to support any new species description (Clark, 1997a).

In general, riboprinting is more practical than rRNA sequence comparisons, as riboprinting is simpler to perform and the banding patterns are simple to interpret (composing of less than 10 fragments). In riboprinting, the long steps of DNA sequence isolation, cloning and sequencing, which can be time consuming and labour intensive and might introduce procedural artefacts, are avoided. Riboprinting also has potentially wide cross-species application since the primers are generated from a well-conserved region in the SSU-rRNA gene (Vaneechoutte *et al.*, 1992). The patterns generated by riboprinting are reproducible and have been shown to be applicable for species differentiation in morphologically similar organisms.

The present study has shown that, in the case of trypanosomes, the riboprinting can detect polymorphism at the species level and sub-species level to a far more limited degree. Therefore, compared to karyotype study by TAFE and RAPD analysis carried out in *T. evansi* stocks in Indonesia, the riboprint technique is less sensitive in detecting stock polymorphisms. It, therefore, has a limited epidemiological value in areas such as Indonesia where there is only one species of Salivarian trypanosomes infecting livestock. The principal need is to distinguish between different stocks with epidemiologically important differences.

CHAPTER SEVEN

GENOMIC FINGERPRINTING OF *TRYPANOSOMA EVANSI* BY SIMPLE SEQUENCE REPEAT-ANCHORED POLYMERASE CHAIN REACTION (SSR-PCR) AMPLIFICATION

7.1. INTRODUCTION

A large proportion of eukaryotic genome consists of repetitive DNA sequences, called satellite DNA, which can be physically separated from the main body of DNA by Caesium Chloride gradient centrifugation. The role and maintenance of such repetitive sequences in the eukaryotic genome, however, are still poorly understood and therefore have attracted the name "junk DNA". The variability within such junk DNA has however proved useful in genetic analysis (Weller, Jeffreys, Wilson *et al.*, 1984) for individual specific DNA fingerprinting of general use in human genetic analysis (Jeffreys, Wilson and Thein, 1985a) including parenthood testing (Jeffreys, Wilson and Thein, 1985b).

Micro and minisatellites, are two classes of satellite DNA, which differ in the number of nucleotide that present in various parts of chromosomes. Extra-chromosomal DNA and DNA from nucleolus are also classified as satellite DNA.

Minisatellites are generally described as variable region of tandem repeats of a short sequence (Jeffreys *et al.*, 1985a) consisting of 30-50 nucleotides in length (Tautz, 1990). In trypanosomes, the presence of minisatellites was first detected by Riou and his co-workers (Riou and Pautrizel, 1977; Riou and Saucier, 1979) and later by Borst *et al.* (1980) in *T. brucei* and *T. equiperdum* after renaturation analysis of non-kinetoplast DNA.

Microsatellites or simple sequences (Tautz and Renz, 1984) are short repetitive sequences, consisting of one to five nucleotides arranged in tandem occurring at high copy numbers at various loci in the genome (Beckman and Weber, 1992). The presence of microsatellites has been detected in all eukaryotic genomes examined from yeast to human (Hamada, Petrino, Kakunaga, 1982).

Microsatellites can be arranged as perfect {e.g. $(CA)_{10}$}, imperfect {e.g. $(CA)_{10}$ ATAT $(CA)_5$} or compound {e.g. $(CA)_{10}$ $(AT)_{10}$} repeats (Kahl, Ramser, Weising *et al.*, 1995). The three types of microsatellites can be found in clusters ('microsatellite islands'), where several microsatellite motifs are mixed and the clusters can be scattered throughout the genome (Kahl *et al.*, 1995). Microsatellites can also undergo dynamic mutations resulting in alleles with varying number of repeat units (Richards and Sutherlands, 1992; Rubinsztein, Amos, Leggo, *et al.*, 1995) and therefore very polymorphic (Kahl *et al.*, 1995). Because of such great variability in their numbers in most loci, microsatellites are suitable as nuclear genetic markers for genome mapping, forensic testing and population studies (Rubinsztein *et al.*, 1995).

The most abundant repeat in eukaryotic genome contains $(CA)_n$. $(GT)_n$ and are frequently referred to as CA repeats (Oliveira *et al.*, 1997). Such CA repeats are scattered in eukaryotic genome, and vary in their copy numbers in different eukaryotic species from 100 copies in yeast to 200,000 copies in salmon (Hamada *et al.*, 1982). Mammals have approximately 50,000-100,000 copies per haploid genome (Hamada *et al.*, 1982) and occur on the average of one every 30 kb in euchromatic regions (Stallings, Ford, Nelson, *et al.*, 1991). *Leishmania* genome is rich in microsatellite sequences, the CA repeats occur one every 50 kb, which is close to the values found in higher eukaryote genome (Rossi, Wincker, Ravel *et al.*, 1994).

7.1.2. APPLICATIONS OF MICROSATELLITES

A number of molecular techniques have been developed that can detect polymorphism among a wide range of species.

7.1.2.1. Microsatellite Fingerprinting

One approaches the detection of microsatellite polymorphisms by microsatellite fingerprinting (Stallings *et al.*, 1991; Bierweth, Kahl, Weigand, 1992; Sharma, Huttel, Winter, *et al.* 1995). Enzyme-restricted genomic DNA is hybridised with oligonucleotides complementary to established microsatellite sequences for the detection of multilocus, restriction fragment length polymorphism. The resulting fingerprints provide for the detection of inter and intra specific variation and can supply information that can be used for taxonomic, phylogenic studies, population dynamics, plant breeding and gene tagging (Kahl *et al.*, 1995). In *Leishmania*, Rossi *et al.* (1994) used three microsatellite probes [$(CA)_n$, $(GT)_n$ and $(GCA)_n$] and showed that the microsatellites are distributed in every chromosomal band between 250-600 kb size range.

7.1.2.2. Microsatellite Primed PCR (MP-PCR)

A different approach to detect microsatellite polymorphisms is to apply a PCR technique using microsatellite sequences used as single primers for amplifying the inter-repeat region. This approach, termed as microsatellite primed polymerase chain reaction (MP-PCR), was first developed by Meyer, Mitchell, Freedman *et al.* (1993). The resulting banding patterns of MP-PCR were similar to that of random amplified polymorphic DNA (RAPD) analysis (Weising, Atkinson, Gardner, 1995; Kahl *et al.*, 1995). MP-PCR has been applied to the detection of inter- and intra-species variation in a wide range of species (Meyer *et al.*, 1993; Weising *et al.*, 1995; Sharma *et al.*, 1995; Baleiras Couto, Hartog, Huis in't Veld *et al.*, 1996, Thanos, Schonian, Meyer *et al.*, 1996; Schonian, Schweynoch, Zlateva, *et al.*, 1996). In MP-PCR, factors that affect RAPD analysis such as primer and $MgCl_2$ concentrations will also influence the resulting MP-PCR banding patterns (Weising *et al.*, 1995; Sharma *et al.*, 1995). Band mismatches can occur in MP-PCR amplification although the reaction is carried out at high stringency conditions (high annealing temperature) (Weising *et al.*, 1995). Sharma *et al.* (1995) improved the PCR specificity by starting the reaction with an annealing temperature (T_A) of well above the expected T_A followed by gradual reduction of the T_A in subsequent cycles. The resulting banding patterns were distinct and reproducible, however, less polymorphic than using a uniform T_A in the PCR amplification (Sharma *et al.*, 1995). It was also suggested (Weising *et al.*, 1995) that primers designed from all dinucleotide and some trinucleotide repeats generated unresolved PCR products as a result from too high numbers of target sites in the genome.

7.1.2.3. Anchored Microsatellite Primed PCR (AMP-PCR) or Simple Sequence Repeat Anchored PCR (SSR-PCR)

The AMP-PCR or SSR-PCR is a variant of MP-PCR developed by Zietkiewics, Rafalski and Labuda, (1994). To overcome the problem of band smearing, which can be present with MP-PCR products, the microsatellites primer used in the AMP-PCR amplification is anchored at the 3' or 5' ends of the primer sequence (Zietkiewics, *et al.*, 1994; Fisher, Gardner and Richardson, 1996, Hantula, Dusabenyagasani and Hamelin, 1996; Oliveira *et al.*, 1997). The AMP-PCR has also been called by several names: Inter-SSR (simple sequence repeat) PCR or SSR-PCR (Zietkiewics, *et al.*, 1994) and RAMS (random amplified microsatellites) (Hantula, *et al.*, 1996).

The primer used by Zietkiewics *et al.* (1994) is a single primer $(CA)_8RY$, where R is any purine and Y is any pyrimidine and the amplification reaction was carried out under high stringency conditions. The banding pattern polymorphism revealed by SSR-PCR assay can

be visualised on a silver-stained polyacrylamide gel (Zietkiewics *et al.*, 1994) or ethidium bromide agarose gel (Hantula *et al.*, 1996).

In the SSR-PCR, anchoring the dinucleotide repeats primers at 3' or 5' ends allows the extension of the primers into the flanking sequence by 2 to 4 nucleotide residues and thus avoids priming from within a $(CA)_n$ repeats (Zietkiewicz *et al.*, 1994). Anchoring, therefore, increases the specificity by reducing the number of targeted genomic loci to those matching the 3' or 5'- terminal residues (Zietkiewicz *et al.*, 1994) and improves band resolution (Oliveira *et al.*, 1997).

Intra-species differences in *T. cruzi* stocks have been demonstrated by SSR-PCR assay and the results were considered to be comparable with those of the RAPD analysis and DNA fingerprinting (Oliveira *et al.*, 1997). The polymorphisms in the SSR-PCR product are not due to the size variations of the target sites, since the primers are anchored at the 3' end (Schonian, *et al.*, 1996; Oliveira *et al.*, 1997) but due to the deletions and insertions in the inter CA regions (Oliveira *et al.*, 1997). These change the sizes of the final DNA segment because the priming site is too distant to support amplification (Schonian, *et al.*, 1996).

The SSR-PCR has advantages over DNA fingerprinting and RAPD analysis (Oliveira *et al.*, 1997). SSR-PCR requires a smaller amount of starting DNA and is much simpler to perform than the DNA fingerprinting. When compared with RAPD analysis, which requires a set of primers, the SSR-PCR only required one primer and one PCR condition for range of different species. The amplification in SSR-PCR is performed at high stringency conditions, which avoids the problem of primer competition for the binding sites that are probably the cause of low reproducibility in the RAPD analysis. The SSR-PCR has been shown to yield good day to day reproducibility and tolerates factors known to affect the reproducibility of RAPD banding patterns, such as variations in DNA and primer concentrations, different brands of *Taq* polymerase and thermocyclers (Oliveira *et al.*, 1997).

7.1.2.4. Random Amplified Microsatellite Polymorphism (RAMP)

Random amplified microsatellite polymorphism (RAMP) is a combination of the RAPD technique and AMP-PCR (Wu, Jones, Danneberger *et al.*, 1994). This method is based on the phenomenon of random distribution of nucleotide sequences immediately flanking simple sequence repeats. RAMP uses primer combinations of arbitrary and labelled microsatellite sequences and the amplification products are resolved in denaturing polyacrylamide gels. The technique is based on the fact that the annealing temperature of the anchored microsatellite primer is 10-15^0 C higher than the RAPD primers. In the amplification reaction with high T_A only the anchored primer should anneal efficiently but at lower T_A cycles both anchored and the RAPD primers should anneal.

7.1.2.5. Sequence Tagged Site Polymorphism

For the generation of sequence tagged microsatellite sites, microsatellite-containing clones are isolated from genomic libraries and the inserts are sequenced in the region around the repeats (Tautz, 1989; Lagercrantz, Ellegren and Anderson, 1993; Rossi *et al.*, 1994; Morchen, Cuguen, Michaelis *et al.*, 1996). PCR primers complementary to the flanking regions are designed and used to amplify the repeat islands, which can show polymorphism because of differences in the size of tandem repeats. This technique was also named as SSLP (simple sequence length polymorphism) (Tautz, 1989). The technique has been used for identity testing, population studies, linkage analysis and genome mapping (Tautz, 1989).

7.1.2.6. Detection of Microsatellite Variants among RAPD Fragments

In this technique the RAPD fragments separated on gels are blotted and hybridised with labelled microsatellite probes. The resulting banding patterns are highly reproducible and polymorphic (Kahl, *et al.*, 1995).

The present study has shown that molecular techniques such as PFGE, RAPD analysis and riboprinting can be used to detect polymorphism in *T. evansi* stocks collected from widely distributed areas in Indonesia. A high degree of polymorphism (46 karyotype patterns) was detected in the karyotype patterns among the stocks tested (80 stocks) by PFGE whereas the RAPD analysis recognised 4 different patterns and riboprinting did not detect any intra-species polymorphism. The polymorphism shown by karyotype and RAPD analysis correlated with the stock isolation locality. The study was carried out to explore the utility of using different genetic markers for *T. evansi* stock characterisation based on microsatellite variation. The PCR-based technique, SSR-PCR used by Oliveira *et al.* (1997) for studying the genetic variability in *T. cruzi*, *Leishmania* and *Schistosoma* was adapted by the present study for use with *T. evansi* stocks from Indonesia.

7.2. MATERIALS AND METHODS

7.2.1. Trypanosomes

All of the DNA samples used were embedded in agarose and the trypanosome stocks used in the study are listed in **Table 4.1**. Trypanosome populations collected from relapse infection (Chapter 4, Section 4.6) and 3 stocks of *T. evansi* from Sudan (TREU 1603/Kassala-4, TREU 1947/Kosti-2, TREU 1733/Hadaliya-61), which are known to be naturally drug resistant (Boid, unpublished results) were also included in the analysis.

7.2.2. Primers

Oligonucleotide primer complementary to microsatellite repeats, $(CA)_8RY$, where R is any purine and Y is any pyrimidine (Oliveira *et al.*, 1997), was synthesised by Cruachem. The primer was diluted to 10 μM with sterile 18.2 MΩ water as working solution and used at 1 μM in the amplification reaction.

7.2..3. SSR-PCR Amplification

The SSR-PCR assay was carried out according to the procedures described by Oliveira *et al.* (1997). Prior to the PCR analysis, working solutions were prepared: a) 10x stock mix of 100 mM Tris-HCl (pH 8.5) and 500 mM KCl; b) 10% (v/v) formamide solution; c) 1 mM working solution of dNTPs from 100 mM stock.

Standard procedures (covering sterile conditions, etc.) for preparing the PCR reactions were employed. Each 50 μl PCR reaction contains 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$, 0.125 mM dNTPs, 2% formamide, 1 μM primer (CA_8RY), 1 unit of *Taq* DNA polymerase (Boehringer Mannheim, Germany) and 10 ng DNA contained in 1/8 agarose block as presented in **Table 7.1**. The PCR amplification was carried out for 26 cycles: denaturation for 30 sec at 94^0 C, annealing for 45 sec at 52^0 C, extension for 1 min at 72^0C then one cycle for 7 min at 72^0C.

Table 7.1. Components of the SSR-PCR amplification reaction.

Component	Volume (μl) per 50 μl reaction	Final concentration
100 mM Tris-HCl (pH 8.5)	5	10 mM
500 mM KCl	5	50 mM
50 mM $MgCl_2$	1.5	1.5 mM
1 mM dNTPs	6.25	0.125 mM
10% formamide	10	2%
10 μM primer $(CA)_8RY$	5	1 μM
Taq polymerase	1 U/ 50 μl	1 U
MilliQ water added to	50 μl	

7.2.4. VISUALISATION OF SSR-PCR AMPLIFICATION PRODUCTS

Amplification products were analysed by electrophoresis in 6% acrylamide gel containing 3 M Urea under non-denaturing conditions (Zietkiewicz *et al.*, 1994). Two different types of gel apparatus were employed, a BioRad Mini gel and a 38 x 50 cm Sequencing gel. Prior use, the glass plates were treated with Bind Silane (γ-methylacryloxypropyltrimethoxysilane) for the longer glass plate (non-bonded) to chemically cross-link the gel to the glass plate. The shorter glass plate (bonded) was treated with Sigmacote to prevent the gel sticking to the plate after electrophoresis.

7.2.4.1. Preparation of the Gel plates

The binding solution to treat the longer of the two glass plates was prepared first by adding 5 µl of Bind Silane to 1 ml 95% ethanol and 5% glacial acetic acid. The plate was cleaned scrupulously and wiped with a paper tissue saturated with 1 ml of freshly prepared binding solution then left for 4-5 min. The plate was then washed by wiping the plate with tissue soaked in 95% ethanol in one direction and then perpendicular to the first direction using gentle pressure. The wash was repeated three times using a fresh paper tissue each wash to remove excess binding solution.

The shorter glass plate was cleaned scrupulously and wiped with a tissue saturated with Sigmacote solution and left for 5 min. Excess of Sigmacote solution was removed by wiping the plate with a paper tissue.

7.2.4.2. Preparation of 6% Acrylamide Gel

7.2.4.2.1. BioRad Mini Gel

The 6% acrylamide gel was prepared by adding 2.7 g Urea in 1.5 ml of 10 x TBE (108 g/L Tris base, 55 g/L Boric acid, 9.3 g/L EDTA) and 2.25 ml of 40% acrylamide/bis-acrylamide (19:1). The volume was adjusted to 15 ml with 18.2 MΩ water and the Urea was dissolved by stirring and gentle heating. The mixture solution was then de-gased under strong vacuum for 5-15 min. After de-gasing, 15 µl of TEMED and 15 µl of 25% (w/v) ammonium persulphate were added to the solution mixture. The gel was cast following the BioRad protocols and left to set for 30-60 min. The Mini Protean II (BioRad) apparatus was assembled for loading. The PCR amplification products were used at 10 µl without any purification and the gel loading buffer (Amersham) was loaded at 2 µl into each of the amplified products. The amplification product mixtures were loaded into the gel at 10 µl per

well. Electrophoresis was carried out at 100 V for 1 hr or until the bromophenol blue tracking dye had reached the bottom of the gel.

7.2.4.2.2. 38 x 50 cm Sequencing Gel

The 6% acrylamide gel was prepared from 27 g Urea in 15 ml of 10x TBE and 22.5 ml of 40% acrylamide/bis-acrylamide (19:1). The volume was adjusted to 150 ml by adding double distilled water. The urea was dissolved by stirring and gentle heating. The mixture was then de-gased under strong vacuum for 5-15 min. After de-gasing, 150 μl of TEMED and 150 μl of 25% ammonium persulphate (APS) were added to the mixture to give a final concentration of 1 μl/ml gel for both TEMED and APS. The gel was cast using the BioRad Sequi-Gen GT protocol and left to set for 30-60 min. Gel loading buffer was added at 10 μl to every 50 μl of PCR product. The PCR product containing the gel loading buffer (Amersham) was then loaded at 10 μl to each well. Electrophoresis was carried out at room temperature at 14V/cm for 10-13 hours in the presence of 1x TBE buffer.

7.2.4.3. Detection

At the end of the electrophoresis, the plates were separated using a wedge leaving the gel affixed to the longer glass plate. The banding patterns were detected by silver staining using a procedure described by Herring *et al.*(1982) as modified by Santos, Pena and Epplen (1993). All incubations were carried out at room temperature with gentle agitation. After electrophoresis, the gel was submerged in freshly prepared fix/stop solution (10% v/v ethanol, 0.5% v/v glacial acetic acid) in a clean plastic tray and incubated for 20 min then the fix solution was discarded. The gel was then rinsed briefly with double-distilled water (ddH_2O). The gel was stained with 0.17% (w/v) Silver Nitrate ($AgNO_3$) and incubated for 25 min. After staining, the gel was then rinsed for 3 min with ddH_2O. The developer solution (3% w/v sodium hydroxide, 0.1% v/v formaldehyde) was then added and incubated with gentle agitation until bands were visible, usually for 5-10 min. After the bands were detected, the reaction was stopped by adding the fix/stop solution. The gel was then rinsed in ddH_2O and documented by photography using a Kodak digital camera interfaced to a BioImage whole band analyser software programme (Millipore, USA).

7.3. RESULTS

The separation of the amplification products using both the BioRad Mini gel and the Sequencing gel systems was poor, with indistinct pattern resolution. The complex banding pattern seen caused great difficulties in the accurate determination of the band sizes,

although the bands of the 1 kb ladder were well resolved. Poor quality of staining along the gel surface and inconsistent appearance of the banding patterns also hindered accurate pattern analysis (**Figures 7.1 and 7.2**).

In an attempt to separate a larger range of band sizes, the electrophoresis was carried out using a standard sequencing gel cast. **Figure 7.3** showed the banding patterns of the amplification products in 24 *T. evansi* stocks separated between ~300-6,000 bp size range. Bands were not detected in the size below 1,000 bp (Lane 2, shows contamination with DNA from the 1 kb ladder).

In general, therefore, the band separation of the amplification products in the sequencing gel did not show any constant results. Some gels (**Figure 7.4 and 7.5**) did not show any banding patterns, although acceptable separation was seen in the 1 kb ladder components in the size range of 500-6,000 bp. **Figure 7.6-7.9** showed the separation of 8 bands in the 1 kb ladder between the size range of 1 to 7 kb. In this case amplification products showed some polymorphic bands, but the band resolution and staining quality were poor, with only one part of the gel sufficiently stained while the other part was not stained properly (**Figure 7.7 and 7.8**).

Inter- and intra-species differences in the SSR-PCR banding patterns were therefore difficult to determine, although some evidence of polymorphism was observed in the banding pattern between the 1.6-10 kb size range (Lanes 2-17 in **Figure 7.9**). The SSR-PCR banding pattern of *T. congolense* stock studied (lane 17 in **Figure 7.9**) was quite different from those of *T. evansi*, characterised by the presence of a band at ~ 4.5 kb in *T. congolense*.

The amplification products of the *T. evansi* population isolated at relapse infections from the same stock (lane 19-25 in **Figure 7.9**) did not show acceptable banding patterns or sufficient quality of staining so detection of any polymorphisms was not possible.

7.4. DISCUSSION

Polymorphisms were not detected in *T. evansi* although a longer run of the electrophoresis was carried out to improve the resolutions of larger bands (between 1000-10000 bp size range). Great technical difficulties were found in the detecting of polymorphisms in the amplification products. Principally, as the gel used to separate the amplification product is very thin (0.04 cm) and large (38x50 cm) and consequently it is difficult to handle. Staining steps have to be carried out with the gel fixed to the glass plate, which could result in uneven staining of the gel surface seen as some part of the gel was not stained and the banding patterns were not hence visible. These banding patterns shown were not easy to analyse, although there appear some banding pattern differences as evidence of inter-specific differences.

As in all PCR-based techniques, the SSR-PCR has the advantage over other methods of genetic analysis in that it does not require a large amount of DNA as starting materials. The technique is theoretically simpler to perform compared to the DNA fingerprinting. In SSR-PCR, high stringency PCR conditions were applied with the aim of avoiding mismatches, or primer competition of binding sites (Meyer *et al.*, 1993), which are factors reported to be responsible for low reproducibility and appearance of artifactual bands in techniques such as RAPD analysis (Penner *et al.*, 1993; Riedy, Hamilton and Aquadro, 1992). The SSR-PCR has been reported as having advantages over the RAPD analysis and DNA fingerprinting, because the SSR-PCR generates good day to day reproducibility in their banding patterns (Oliveira *et al.*, 1997).

Although the SSR-PCR had been shown (Oliveira *et al.*, 1997) to be a potentially useful technique for studying genetic variability of different parasites species, this study has shown that its reliability to detect polymorphisms in *T. evansi* stocks remains unconfirmed due to technical problems. This study has suggested that the use of SSR-PCR for characterisation of *T. evansi* still needs standardisation, especially on the detection technique, which includes the electrophoresis and staining methods.

Figure 7.1. Banding patterns shown by *T. evansi* stocks which were subjected to simple sequence repeat-anchored polymerase chain reaction amplification with the $(CA)_8$ RY primer. The amplification products were detected by an acrylamide gel electrophoresis using MiniProtean II gel electrophoresis apparatus (Biorad).

Tracks 1, 2: 1 kb DNA standard size marker
Track 4: BAKIT 429
Track 5: BAKIT 431
Track 6: BAKIT 434
Track 7: BAKIT 437
Track 8: BAKIT 439
Track 9: BAKIT 444
Track 10: BAKIT 445
Track 11: BAKIT 446
Track 12: BAKIT 461
Track 13: BAKIT 463

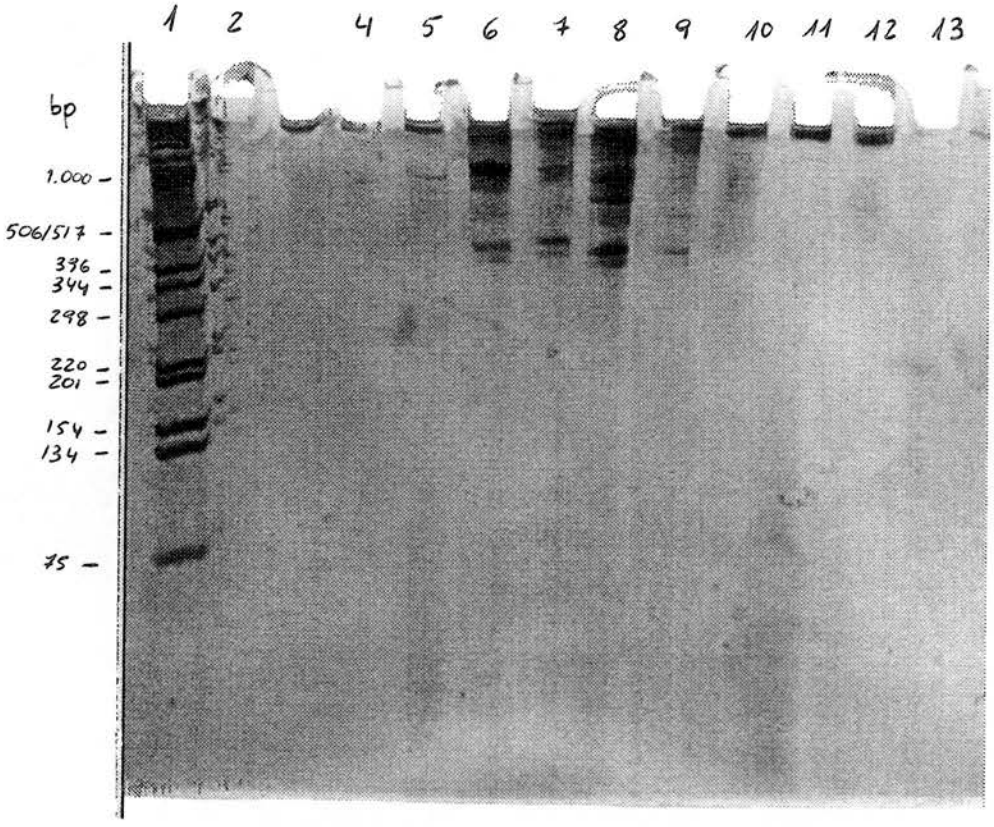

1 2 4 5 6 7 8 9 10 11 12 13
bp
1.000
506/517
396
344
298
220
201
154
134
75

Figure 7.2. Banding patterns shown by *T. evansi* stocks which were subjected to simple sequence repeat-anchored polymerase chain reaction amplification with the $(CA)_8$ RY primer. The amplification products were detected by an acrylamide gel electrophoresis using MiniProtean II gel electrophoresis apparatus (Biorad).

Track 1: 1 kb DNA standard size marker
Track 3: BAKIT 505
Track 4: BAKIT 508
Track 5: BAKIT 509
Track 6: BAKIT 510
Track 7: BAKIT 511
Track 8: BAKIT 512
Track 9: BAKIT 513
Track 10: BAKIT 514
Track 11: BAKIT 517
Track 12: BAKIT 519
Track 13: BAKIT 380
Track 14: BAKIT 383

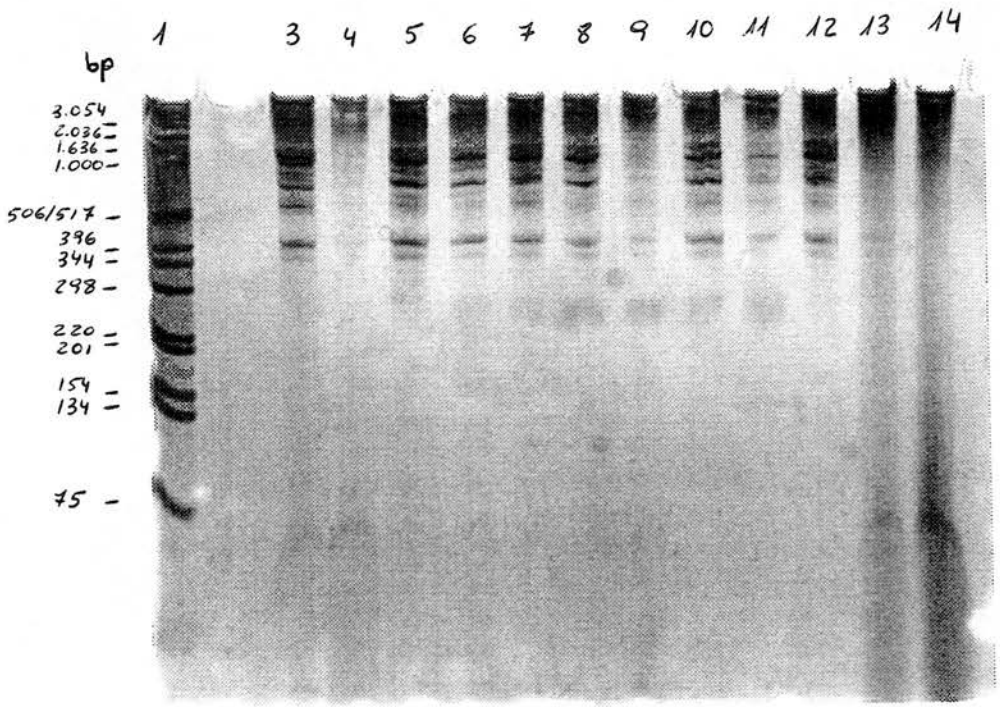

1
3
4
5
6
7
8
9
10
11
12
13
14
bp
3.054
2.036
1.636
1.000
506/517
396
344
298
220
201
154
134
75

Figure 7.3. *T. evansi* banding patterns after amplification with the $(CA)_8RY$ primer which were separated in a 6% acrylamide gel using a standard sequencing gel electrophoresis.

Track 1: 1 kb DNA standard size marker
Track 2: BAKIT 392
Track 3: BAKIT 393
Track 4: BAKIT 394
Track 5: BAKIT 399
Track 6: BAKIT 400
Track 7: BAKIT 401
Track 8: BAKIT 402
Track 9: BAKIT 403
Track 10: BAKIT 409
Track 11: BAKIT 410
Track 12: BAKIT 414
Track 13: BAKIT 413
Track 14: BAKIT 415
Track 15: BAKIT 416
Track 16: BAKIT 417
Track 17: BAKIT 421
Track 18: BAKIT 422
Track 19: BAKIT 423
Track 20: BAKIT 424
Track 21: BAKIT 425
Track 22: BAKIT 426
Track 23: BAKIT 427
Track 24: BAKIT 428
Track 25: BAKIT 429

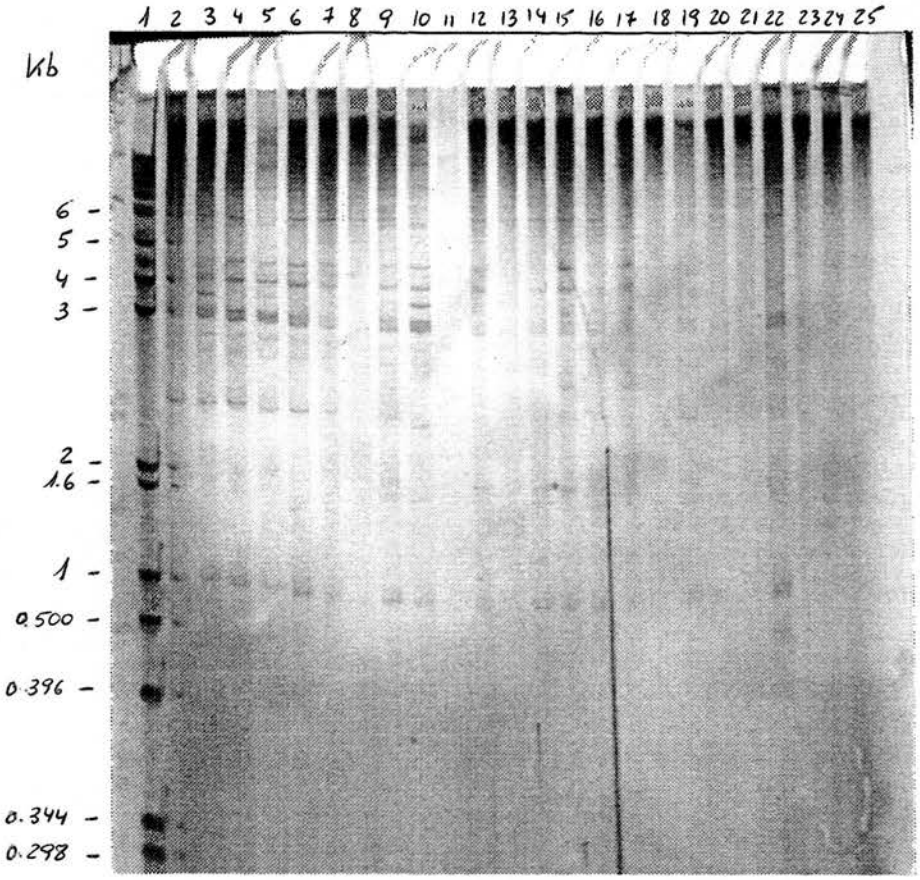

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
kb
6
5
4
3
2
1.6
1
0.500
0.396
0.344
0.298

Figure 7.4. The banding patterns in *T. evansi* stocks after being subjected to the simple sequence repeat-anchored PCR. The electrophoresis was carried out to separate bands in the 500-6,000 bp size range.

Track 1: 1 kb DNA standard size marker
Track 2: BAKIT 392
Track 3: BAKIT 393
Track 4: BAKIT 394
Track 5: BAKIT 399
Track 6: BAKIT 400
Track 7: BAKIT 401
Track 8: BAKIT 402
Track 9: BAKIT 403
Track 10: BAKIT 409
Track 11: BAKIT 410
Track 12: BAKIT 414
Track 13: BAKIT 413
Track 14: BAKIT 415
Track 15: BAKIT 416
Track 16: BAKIT 417
Track 17: BAKIT 421
Track 18: BAKIT 422
Track 19: BAKIT 423
Track 20: BAKIT 424
Track 21: BAKIT 425
Track 22: BAKIT 426
Track 23: BAKIT 427
Track 24: BAKIT 428
Track 25: BAKIT 429

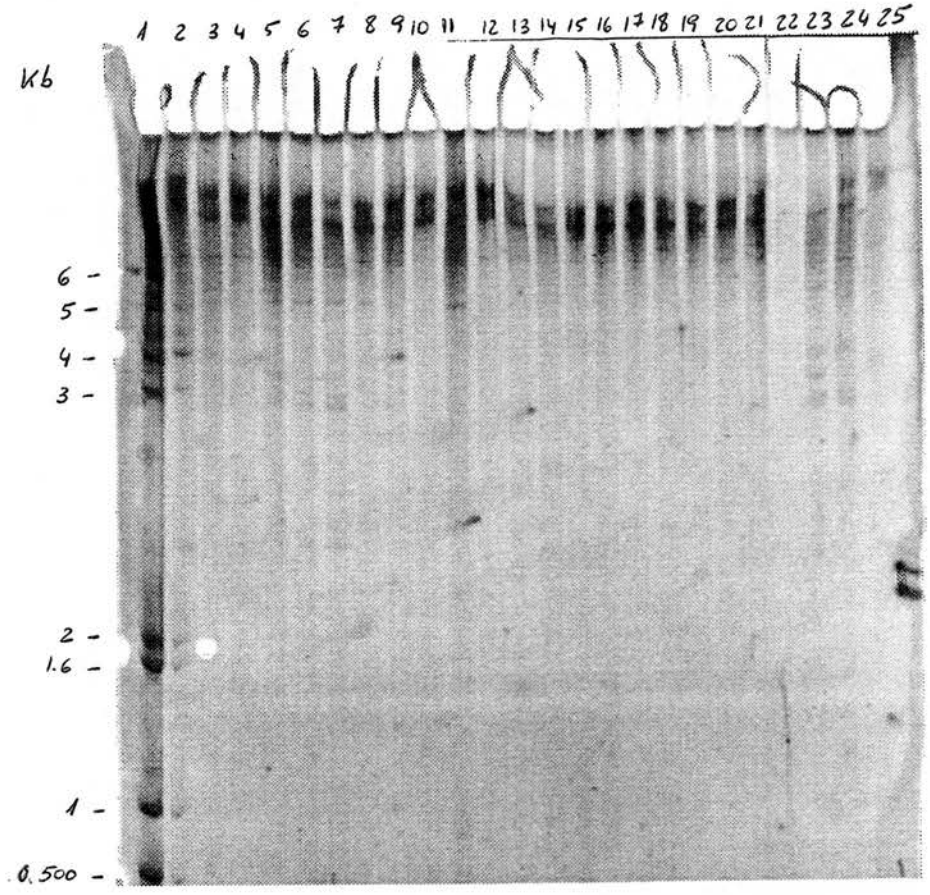

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Kb
6 -
5 -
4 -
3 -
2 -
1.6 -
1 -
0.500 -

Figure 7.5. Banding patterns in *T. evansi* stocks after being subjected to the simple sequence repeat-anchored PCR. The electrophoresis was carried out to separate bands in the 500-6,000 bp size range.

Track 1: 1 kb DNA standard size marker
Track 2: BAKIT 100
Track 3: BAKIT 126
Track 4: BAKIT 134
Track 5: BAKIT 148
Track 6: BAKIT 229
Track 7: BAKIT 251
Track 8: BAKIT 254
Track 9: BAKIT 294
Track 10: BAKIT 296
Track 11: BAKIT 312
Track 12: BAKIT 362
Track 13: BAKIT 371
Track 14: BAKIT 372
Track 15: BAKIT 373
Track 16: BAKIT 374
Track 17: BAKIT 375
Track 18: BAKIT 381
Track 19: BAKIT 382
Track 20: BAKIT 383
Track 21: BAKIT 384
Track 22: BAKIT 385
Track 23: BAKIT 386
Track 24: BAKIT 388
Track 25: BAKIT 390

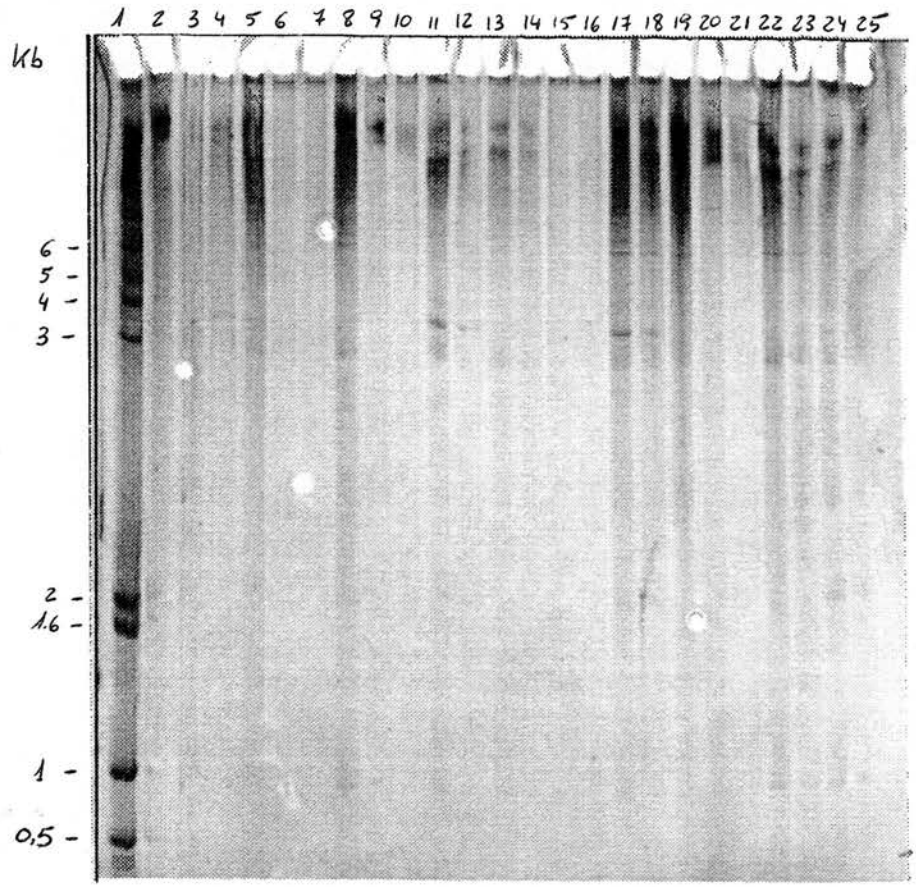

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
kb
6
5
4
3
2
1.6
1
0.5

Figure 7.6. Variations in the banding patterns (between the 1-7 kb size range) shown by *T. evansi* stocks amplified with the $(CA)_8RY$ primer.

Track 1: 1 kb DNA standard size marker
Track 2: BAKIT 100
Track 3: BAKIT 126
Track 4: BAKIT 371
Track 5: BAKIT 134
Track 6: BAKIT 148
Track 7: BAKIT 229
Track 8: BAKIT 251
Track 9: BAKIT 254
Track 10: BAKIT 294
Track 11: BAKIT 296
Track 12: BAKIT 312
Track 13: BAKIT 362
Track 14: BAKIT 372
Track 15: BAKIT 373
Track 16: BAKIT 374
Track 17: BAKIT 375
Track 18: BAKIT 381
Track 19: BAKIT 381
Track 20: BAKIT 384
Track 21: BAKIT 385
Track 22: BAKIT 386
Track 23: BAKIT 388
Track 24: BAKIT 390

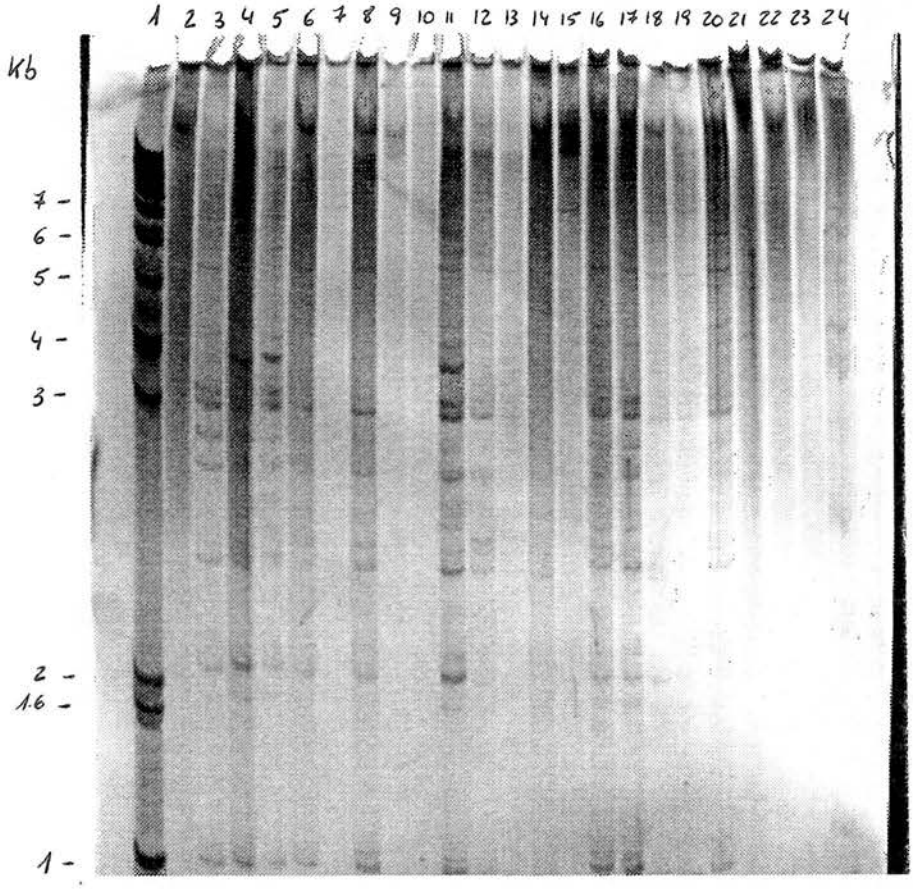

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Kb
7
6
5
4
3
2
1.6
1

Figure 7.7. Banding patterns of *T. evansi* stocks amplified with the $(CA)_8RY$ primer.

Track 25: 1 kb DNA standard size marker
Track 1: BAKIT 510
Track 2: BAKIT 509
Track 3: BAKIT 508
Track 4: BAKIT 505
Track 5: BAKIT 504
Track 6: BAKIT 503
Track 7: BAKIT 502
Track 8: BAKIT 500
Track 9: BAKIT 499
Track 10: BAKIT 498
Track 11: BAKIT 496
Track 12: BAKIT 482
Track 13: BAKIT 475
Track 14: BAKIT 467
Track 15: BAKIT 463
Track 16: BAKIT 461
Track 17: BAKIT 446
Track 18: BAKIT 445
Track 19: BAKIT 444
Track 20: BAKIT 439
Track 21: BAKIT 437
Track 22: BAKIT 435
Track 23: BAKIT 434
Track 24: BAKIT 431

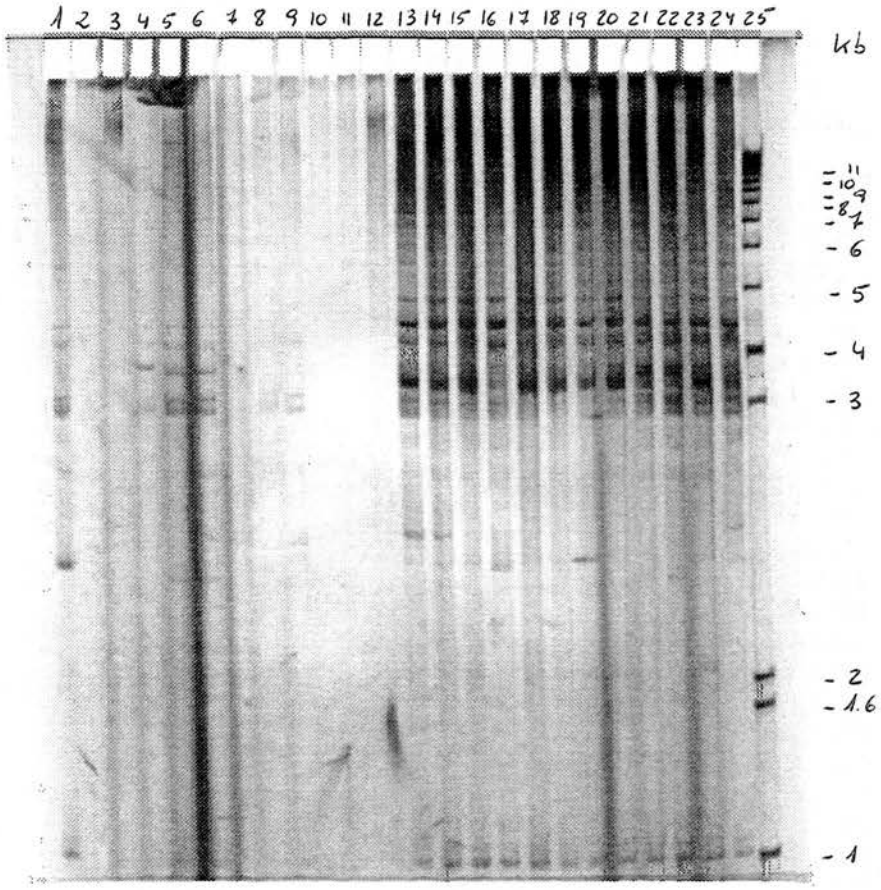

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
kb
11
10
9
8
7
6
5
4
3
2
1.6
1

Figure 7.8. Polyacrylamide gel used to detect the banding patterns separation of *T. evansi* stocks amplified with the $(CA)_8RY$ primer between 500-9,000 bp size range illustrates the staining problem experienced in SSR-PCR carried out in the present study.

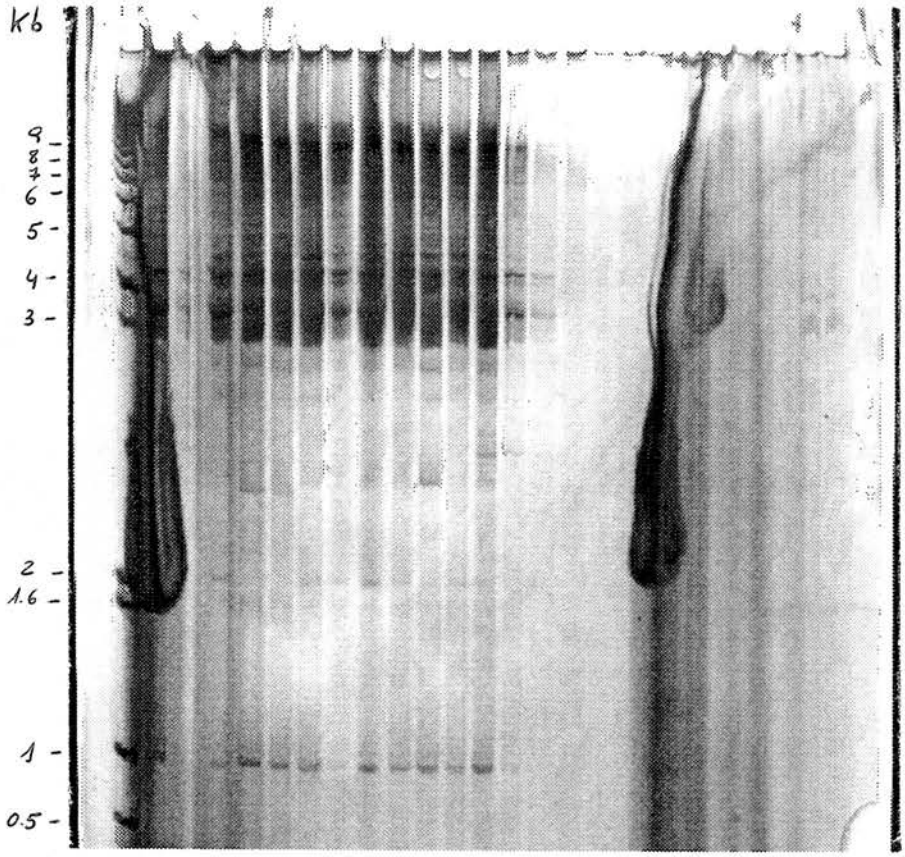
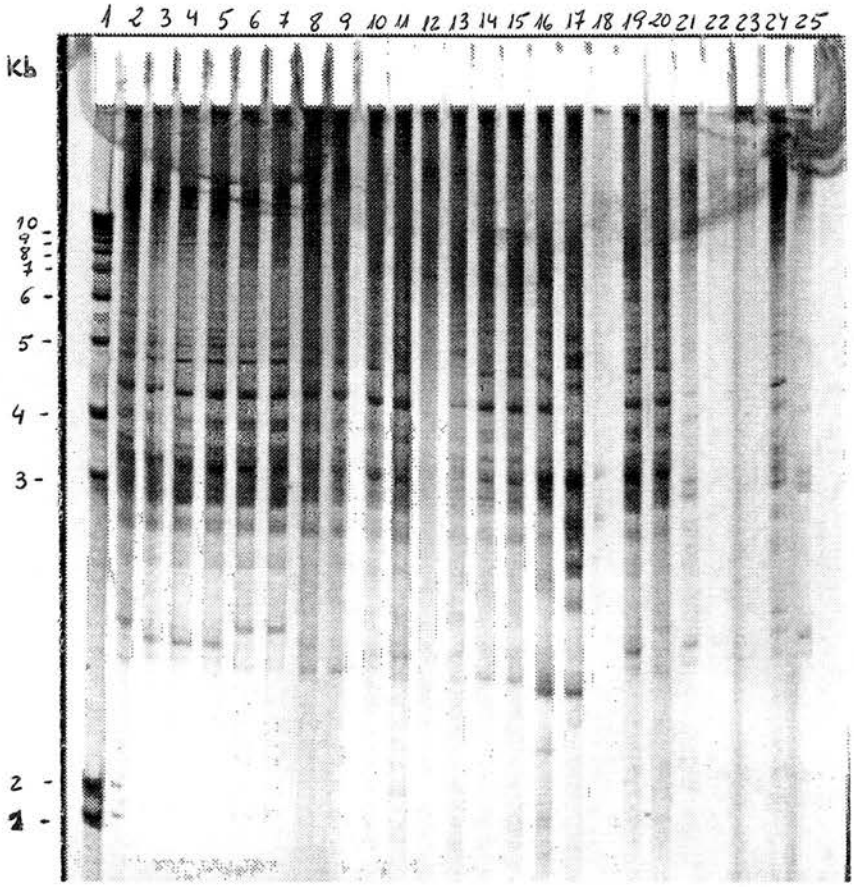

kb
9
8
7
6
5
4
3
2
1.6
1
0.5

Figure 7.9. Banding pattern variations of the *T. evansi*, *T. brucei* and *T. congolense* (TREU 2193) stocks amplified with the $(CA)_8RY$ primer in the size range of 1.6 to 10 kb.

Track 1: 1 kb DNA standard size marker
Track 2: BAKIT 511
Track 3: BAKIT 512
Track 4: BAKIT 513
Track 5: BAKIT 514
Track 6: BAKIT 517
Track 7: BAKIT 519
Track 8: BAKIT 380
Track 9: BAKIT 383
Track 10: TREU 1810 (*T. evansi*, Kenya)
Track 11: TREU 2187 (*T. evansi*, Brazil)
Track 12: TREU 1603
Track 13: TREU 1947
Track 14: BAKIT 389
Track 15: BAKIT 387
Track 16: TREU 2177 (*T. brucei*)
Track 17: TREU 2193 (*T. congolense*)
Track 18: TREU 1733
Track 19: TREU 2253
Track 20: TREU 2232
Track 21: TREU 2261
Track 22: BAKIT 374
Track 23: TREU 2257
Track 24: TREU 2256
Track 25: TREU 2258

} *T. evansi* Relapse populations


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Kb
10
9
8
7
6
5
4
3
2
1

CHAPTER EIGHT

GENERAL DISCUSSION

One of the primary processes in understanding parasite epidemiology is the ability to correlate disease and its causative organism. This is particularly problematical in organisms such as trypanosomes that exist as morphologically identical populations but exhibit widely different properties such as pathogenicity. The ability to relate the degree of diversity among trypanosome stocks to features of epidemiological significance is therefore an important step in achieving a better understanding of the parasite epidemiology. *Trypanosoma evansi* has the most widespread geographical distribution of the trypanosome species affecting livestock and infects a range of hosts with different consequences which suggests that *T. evansi* may be consist of a range of genotypes. The most specific and direct method for characterising *T. evansi* isolates lies at the genetic level based on the analysis of DNA and RNA sequences. Although differences in DNA composition of a trypanosome stock or species can be expressed as phenotypic characteristics such as differences in the infectivity to vertebrate hosts, morphology, life cycle and sensitivity to trypanocidal drugs, DNA-based techniques offer the advantage of directly analysing trypanosome DNA instead of relying on indirect methods based on phenotypic characteristics which often result from changes in several genes. The present study used a range of techniques - karyotype analysis, random amplified polymorphic DNA (RAPD) analysis, Riboprinting and simple sequence repeat polymerase chain reaction (SSR-PCR) to examine genetic variation in stocks of Indonesian *T. evansi* with the aim of identifying molecular markers of epidemiological significance.

Overall, the results from the present study suggested that, of the techniques used, karyotyping and RAPD analysis had the greatest potential for characterising *T. evansi* stocks collected from Indonesia. Karyotyping was more sensitive than the RAPD analysis in that it could detect a greater degree of inter-stock variation than RAPD, while RAPD analysis was more suitable for the rapid characterisation of a large number of samples. Riboprinting was less sensitive in detecting variability among *T. evansi* stocks than either karyotyping or RAPD analysis while technical difficulties prevented the thorough investigation of SSR-PCR as a potentially novel method of assessing variation among *T. evansi* stocks.

Karyotype analysis of *T. evansi* stocks was carried out using the transverse alternating field electrophoresis (TAFE) system and a range of electrophoretic running conditions were developed in order to resolve DNA fragments across different size ranges. The TAFE system used in the present study produced sharp, well resolved and reproducible bands in every electrophoretic run. The electrophoresis time is relatively short (18-36 hrs) for separating DNA fragments smaller than 1.6 Mb, however, the separation of larger DNA molecules need considerably longer running times (3-6 days). The TAFE system as developed in this study was able to separate *T. evansi* DNA into a total of 17 to 27 chromosomal units in the size range of 50 kb to 5.7 Mb according to stock. The exact numbers of chromosomes in each electrophetically-separated band in *T. evansi*, however, was difficult to estimate due to non-stoichiometric ethidium bromide staining with the DNA bands. For example, a band of 30 kb could give the same fluorescence with a band of 300 kb because the 30 kb band might consist of 10 chromosomes each of 30 kb. Complete resolution of all potential chromosome bands of *T. evansi* could not be achieved even using the wide range of TAFE running conditions developed in the present study. Because of technical limitations and the wide range of chromosome sizes in trypanosomes the complete separation of all chromosomes is not possible (Henriksson *et al.*, 1995) and consequently, complete resolution of the karyotype pattern has not been achieved by PFGE with any trypanosome stock (Swindle and Tait, 1996). The typical chromosomal banding patterns of *T. evansi* separated by TAFE was organised into four regions: 1) chromosomal DNA that remained in the sample well and was unresolved under the particular electrophoresis conditions used 2) an undetermined number of large chromosomes (>1,000 kb) and chromosomes that stayed in the compression zone of the gel 3) several (2 to 8) intermediate sized chromosomes (200-900 kb) and 4) a highly variable number of minichromosomes (50-150 kb). The potential number of chromosomal bands in each size class is unknown but the numbers detected differ from one stock to another. Karyotype organisation patterns in *T. evansi* stocks different from those seen in the present study have been reported by other workers, for example, Waitumbi *et al.* (1994) and probably reflect technical differences in equipment used and the separation conditions. The present study used a Beckman TAFE system optimised to separate *T. evansi* chromosomal DNA in the 50-900 kb size range while Waitumbi *et al.* (1994) used CHEF system optimised to separate chromosomal bands in the size range of 50-600 kb. These technical differences probably resulted in more intermediate-sized chromosomal bands (200-900 kb) being detected by the present study.

The eighty *T. evansi* stocks used in the present study showed a high degree of polymorphism in the karyotype patterns with 46 different patterns being detected. These 46 patterns were grouped into seven main karyotype groups, which were associated with particular areas and provided evidence of geographical isolation of stocks. The high degree of polymorphism in the karyotype patterns found in the *T. evansi* stocks in this study was markedly in contrast with the

limited variability found in *T. evansi* stocks from Kenya (Waitumbi and Young, 1994) and China (Lun *et al.*, 1992b).

The principal factor contributing to the variability in the karyotype banding patterns is the size differences of individual chromosome elements. In *T. brucei*, changes in size have been attributed to the activation of VSG gene expression, which involves transposition of hundreds of kilobase pairs of DNA from one chromosome to another resulting in changes in chromosomal banding patterns (Van der Ploeg *et al.*, 1984a; Shea *et al.*, 1986). However, in the present study the changes in chromosome banding patterns could not be related to changes in the variable antigenic types. Karyotype banding pattern variability could be caused by changes in the size of chromosomal elements in trypanosomes, such as *T. brucei*, resulting from genetic exchange during its sexual stage in the tsetse vector (Gibson and Garside, 1991; Tait *et al.*, 1996). However, the occurrence of such genetic exchange in *T. evansi* has not been described but cannot be excluded. The high degree of karyotype variability seen in the *T. evansi* stocks studied could arise as a consequence of rapid asexual division during infection as evidence has been presented that links high degree of diversity with organisms that reproduces by rapid asexual division (Mayr, 1963) as do trypanosomes. Furthermore, the insular nature of the Indonesian archipelago might result in the emergence of geographically isolated of stocks which develop their own karyotype patterns. Some evidence for this was obtained in the present study as some banding patterns were associated with particular localities. However, extensive animal movements between islands are likely to obscure such patterns. Evidence for the stability of molecular karyotype patterns was found in the present study in that stocks from West and Central Java showed a high degree of karyotype patterns stability during a six-year period from 1988-1994. This stability of karyotype patterns suggests that once a particular karyotype pattern emerges in response to local selection pressures then that pressure should serve to stabilise the pattern within that area. The introduction of trypanosome stocks with different karyotype into Indonesia on different occasions might also explain the variation seen in the present study but this would depend on the presence of multi-karyotype populations outside Indonesia. However, there is no information available about the variability in the karyotype of *T. evansi* in other countries in Southeast Asia. In addition to the factors mentioned above, the high degree of variability in the karyotype banding pattern in Indonesia might be correlated with the perceived historical origin of *T. evansi* strains. The history of the introduction of surra to the Indonesian archipelago is considered different from its introduction to Kenya and China. Hoare (1972) postulated that *T. evansi* evolved from *T. brucei* by adaptation to mechanical means of transmission when caravans of camels came into contact with *T. brucei* and then returned to tsetse-free areas. Lun *et al.* (1992b) suggested that *T. evansi* entered China by multiple introductions of *T. evansi* via the camel trade to north-west China in addition to the introduction of infected mules from Burma (Luckins, 1988). In Indonesia *T. evansi* might have entered by

transmission of the parasite from transported animals from India (Dieleman, 1983) at the beginning of the 20th century, when India was heavily infested with surra (Hoare, 1972). However, the introduction of *T. evansi* to Indonesia might have been started a long time before it was first reported by Penning in 1903. Livestock could have been sporadically brought into the country since the establishment of an agricultural system in the 8th century. Traders from India (15th century), Portugal (16th century) and the Netherlands (17th century) might have brought livestock in to Indonesia, for example, horses for their cavalry, cattle for their trade. Some of these animals might have been infected during the stopovers and suffered from subclinical *T. evansi* infection, which eventually transmitted among local animals. This multiple introduction of *T. evansi*, possibly consisting of mixed karyotypes, might contribute to the high degree of variability found in the karyotype patterns of *T. evansi* stocks in Indonesia.

The practical application of karyotype analysis of *T. evansi* stocks by PFGE was also shown from results with stocks from particular groups of animals. Results from studies on buffaloes from Central Java to North Sumatra transported showed PFGE could be used to study transmission dynamics of *T. evansi* between local and imported animals. In this case it was shown that *T. evansi* infection had been transferred from the local to the transported buffaloes, not vice-versa as the karyotype of local stocks differed sufficiently from those found in the areas from where the animals had been exported.

Conversely, the high degree of similarity in karyotype pattern found in *T. evansi* stocks isolated from a group of Bali-cattle kept together in a feedlot in Lampung suggested that an outbreak of surra in these animals might be attributable to the introduction of a single, highly virulent stock which displaced an existing stock. Similarly, a mono-karyotyc pattern was also found in stocks isolated from cattle and buffaloes during a surra outbreak in Madura.

The use of random amplification polymorphic DNA (RAPD) techniques also revealed a degree of genetic diversity between stocks of *T. evansi* from Indonesia, although not as marked as that shown by PFGE. Using the primer ILO525 originally applied for intra-specific identification of *T. vivax* (Dirie *et al.*, 1993b), the present study detected two different patterns among the nine Indonesian stocks studied. Previously, this primer failed to demonstrate any polymorphism in the RAPD pattern among the Kenyan stocks (Waitumbi and Murphy, 1993), and supports the finding from PFGE analysis that Indonesian stocks of *T. evansi* are genetically more diverse than those from Kenya. However, the different results obtained in the two studies might be due by factors that influencing the fidelity in the RAPD analysis such as Mg^{++} concentrations, primer concentrations, enzyme source and concentration, DNA extraction methods, DNA concentration, the model of thermocycler used and inter-laboratories condition (Tyler *et al.*, 1997). Only two conditions in the RAPD analysis carried out in the present study, however, were different from those of Waitumbi and Murphy (1993) namely the source of the DNA polymerase and the model

of the thermocycler but the impact of these could not be assessed as the stocks used by Waitumbi and Murphy (1993) were not available to the present study.

Four different RAPD patterns were detected among the 80 Indonesian stocks using the randomly generated 10-mer primer GEN-046. These patterns correlated with the locality from where the stocks were isolated. A degree of correlation was also seen between PFGE karyotype patterns and RAPD pattern (**Table 8.1**) e.g. the 13 *T. evansi* stocks from Lampung with identical karyotype patterns (karyotype pattern 17, group 1.3) have two different RAPD patterns (pattern 1 and 4). This suggests that the RAPD analysis and karyotyping are detecting variation within different system. Karyotype analysis determines the size classes of individual chromosomes, which differ between different stocks that may or may not affect the sites to which the primers attach, whereas RAPD analysis detects polymorphisms of random DNA sequences in the genome which are unlikely to affect chromosome size. Furthermore the random nature of RAPD primer binding sites makes it difficult to provide a functional explanation for the RAPD patterns found among the *T. evansi* stocks. Nevertheless, the fact that a degree of agreement between results from karyotyping and RAPD analysis was seen provides evidence that the stock grouping are taxonomically valid although their epidemiological and functional significance remains largely unknown.

Riboprinting of the *T. evansi* used in the present study revealed a more limited genetic variation than either PFGE or RAPD. The riboprinting technique used in the present study did not detect any differences in post-restriction-digested banding patterns between the Indonesian stocks. However, riboprinting was able to differentiate the stocks from Indonesia and China from those isolated in Kenya which suggests either a separate origin for African and Asian stocks or that the African stocks have been isolated from the Asian stocks for sufficient time to allow the two populations to accumulate their own distinctive set of mutations within their ribosome genes. Riboprinting has been used to detect intra-specific variation among *T. cruzi* isolates that has been correlated with the host from which they were isolated (Clark and Pung, 1994) and is probably a reflection of the well documented greater diversity seen between isolates of *T. cruzi*. Differences in riboprint patterns arise from mutations in the variable regions of the rRNA gene that result in the appearance of or disappearance of DNA sequences that are recognised by restriction. Therefore, riboprinting can only detect changes in a small part of the gene sequence and significant variations in the sequence in other parts will not be detected.

Table 8.1. Comparison between karyotype group and RAPD patterns detected in the 80 *T. evansi* stocks from Indonesia (Summarised from dendrograms in Figure 4.9 and 5.16).

Stock No. (BAKIT)	Karyotype Pattern	Karyotype Group	RAPD Pattern	Isolation locality
371	1	1.1	2	Central Java
386	2	1.1	2	North Sumatra
134	3	1.1	2	West Java
373	4	1.1	1	Central Java
375	4	1.1	1	Central Java
505	5	1.1	2	West Java
374	6	1.2	1	Central Java
508	6	1.2	1	Central Java
509	6	1.2	1	Central Java
512	6	1.2	1	Central Java
513	6	1.2	4	Central Java
514	6	1.2	1	Central Java
498	6	1.2	1	West Java
499	6	1.2	1	West Java
502	6	1.2	1	West Java
435	7	1.2	3	Lampung
437	7	1.2	3	Lampung
426	8	1.2	1	North Sumatra
428	8	1.2	1	North Sumatra
511	9	1.2	4	Central Java
362	10	1.2	1	Madura
517	10	1.2	1	Madura
519	10	1.2	1	Madura
251	11	1.2	1	North Sulawesi
403	12	1.2	1	South Kalimantan
148	13	1.2	1	North Sulawesi
254	14	1.2	1	South Sulawesi
385	15	1.3	1	North Sumatra
100	16	1.3	1	North Sulawesi
410	17	1.3	1	Lampung
411	17	1.3	4	Lampung
413	17	1.3	1	Lampung
415	17	1.3	1	Lampung
416	17	1.3	1	Lampung
417	17	1.3	1	Lampung
421	17	1.3	1	Lampung
434	17	1.3	1	Lampung
439	17	1.3	1	Lampung
444	17	1.3	1	Lampung
445	17	1.3	4	Lampung
446	17	1.3	4	Lampung
463	17	1.3	4	Lampung

380	18	1.4	2	North Sumatra
382	18	1.4	3	North Sumatra
384	19	1.4	2	North Sumatra
390	20	1.4	2	North Sumatra
388	21	1.4	2	North Sumatra
429	22	1.4	2	North Sumatra
383	23	1.4	2	North Sumatra
401	23	1.4	2	North Sumatra
389	23	1.4	2	North Sumatra
387	24	1.4	2	North Sumatra
402	25	1.4	2	North Sumatra
394	26	1.4	2	North Sumatra
393	27	1.4	2	North Sumatra
422	28	1.5	2	North Sumatra
423	28	1.5	3	North Sumatra
510	29	1.5	2	Central Java
312	30	1.5	1	Aceh
467	31	1.5	1	East Java
475	31	1.5	1	East Java
496	31	1.5	4	East Java
482	32	1.5	1	East Java
381	33	1.6	2	North Sumatra
431	34	1.6	1	Lampung
400	35	1.6	2	North Sumatra
392	36	1.6	2	North Sumatra
409	37	1.6	1	Lampung
296	38	1.6	2	South Sulawesi
461	39	1.6	2	South Kalimantan
372	40	2	1	Central Java
424	40	2	1	North Sumatra
427	40	2	1	North Sumatra
425	40	2	1	North Sumatra
503	41	2	1	West Java
504	42	2	1	West Java
399	43	2	3	North Sumatra
294	44	2	4	West Java
500	45	2	2	West Java
126	46	2	2	East Java

The simple sequence repeat PCR (SSR-PCR) has great potential for detecting genetic variability in different parasite species with results considered to be comparable with those from RAPD analysis and DNA fingerprinting (Oliveira *et al.*, 1997). SSR-PCR requires smaller amount of DNA than the fingerprinting and is in some ways simpler to perform than RAPD analysis, as SSR-PCR only requires one primer and one PCR condition for a range of different DNA template species. However, SSR-PCR used in the present study failed to detect any differences between the *T. evansi* stocks included in the study. Differences in the SSR-PCR banding patterns arise from dynamic mutations of simple sequence repeats or microsatellites which produce alleles with

varying number of repeat units (Richards and Sutherlands, 1992; Rubinsztein *et al.*, 1995). On a practical note, great technical difficulties were found in reliably detecting any polymorphisms arising from the need to identify and analyse the large number of bands produced by the amplification procedure and the subsequent analysis of the banding patterns from the amplification products. In the present study the SSR-PCR technique, however, was able to differentiate between unrelated trypanosome species in that *T. congolense* could be distinguished from *T. brucei*-group organisms (*T. brucei* and *T. evansi*). At present the technical difficulties in carrying out SSR-PCR probably reduce its potential usefulness for strain identification.

Overall, therefore, the principal result from the present study has shown that extensive genetic diversity exists among the *T. evansi* stocks studied and in particular those from Indonesia which is in marked contrast to findings with stocks from other parts of the world. The reason for such marked diversity is unclear as is the epidemiological significance or potential of such variation. Clearly a highly diverse population is better able to respond much quicker to selection pressures such as the use of drugs than a more homogeneous population. The high degree of diversity among Indonesian isolates might be a reflection of its relatively recent introduction into the archipelago but may also linked to geographical isolation within an archipelago system or even the existence of a hitherto undescribed vector or host system within which the parasite undergoes sexual reproduction. The study has also highlighted the potential problems in the application of molecular-based assays in studies on organisms such as trypanosomes with a wide spectrum of discriminatory power seen with the range of techniques employed. The PFGE technique based on the separation of chromosome-sized pieces of DNA provided the greatest degree of discrimination between individual stocks of *T. evansi* while the least discriminatory was a SSR-PCR technique which could only differentiate between widely separated trypanosome species. Between these extremes RAPD could differentiate between stocks from Indonesia with a degree of correlation with PFGE results while riboprinting could differentiate between Asian and African stocks of *T. evansi*.

The present study represents the most extensive study on genetic diversity in *T. evansi* stocks in Southeast Asia carried out to date. It has shown that stocks from *T. evansi* show a much greater genetic diversity than those that have been examined from other endemic areas. The present study also has highlighted the most appropriate techniques for use in further studies to explore the significance of this diversity, which could include virulence and drug sensitivity as well as extending studies to *T. evansi* stocks from other areas in Asia, Africa and South America.

Overall, the present study showed that the molecular techniques such as karyotyping, RAPD analysis and riboprinting are useful to detect inter-stock genetic variation of *T. evansi* that can be related to epidemiologically important factors such as geographical origin of infections. Further studies need to be done, however, to identify molecular markers, such as DNA probes, that could

be used for a better understanding on the epidemiological features of *T. evansi* such as the presence of any drug resistant strains, their origin in particular hosts which they were isolated and their pathogenicity.

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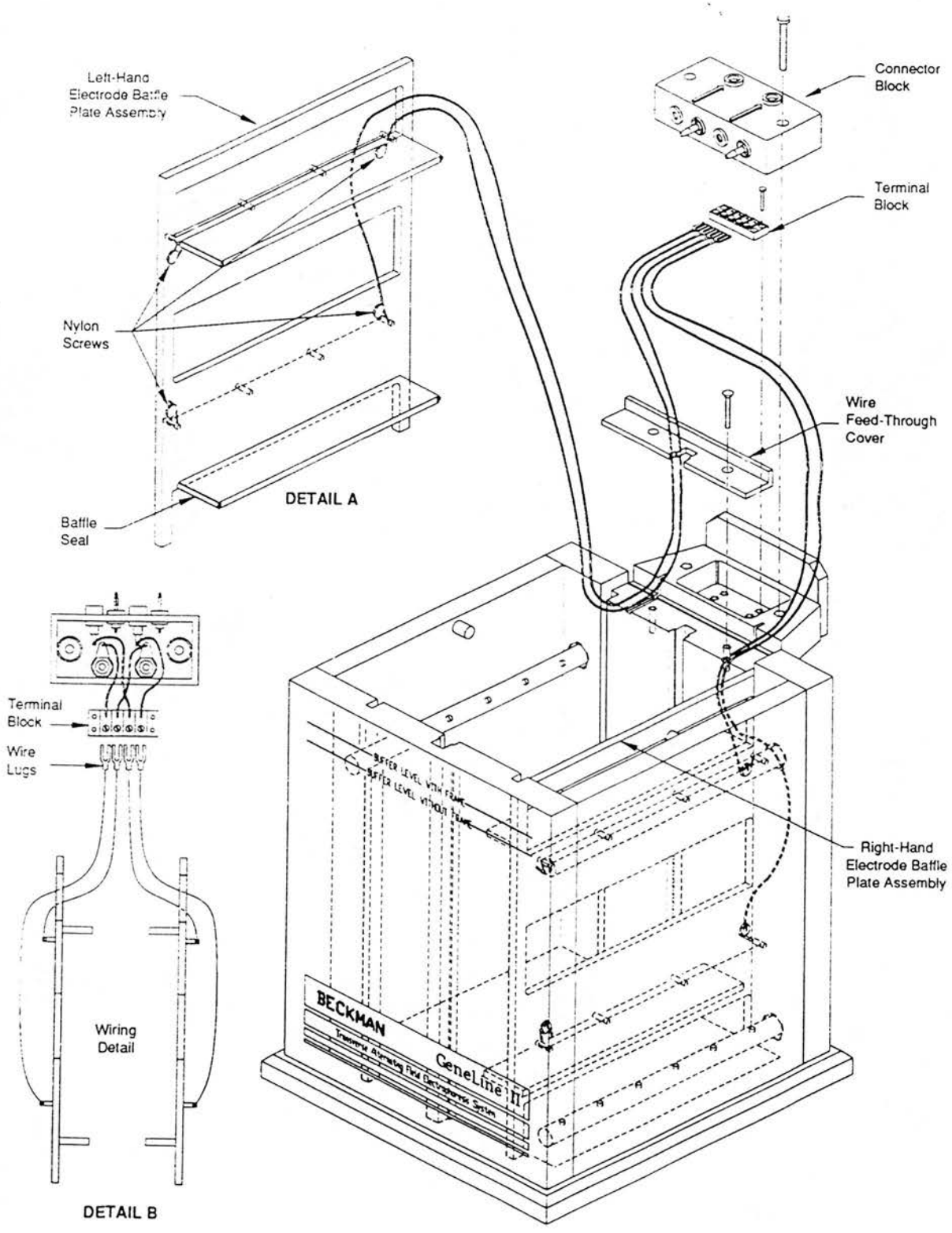
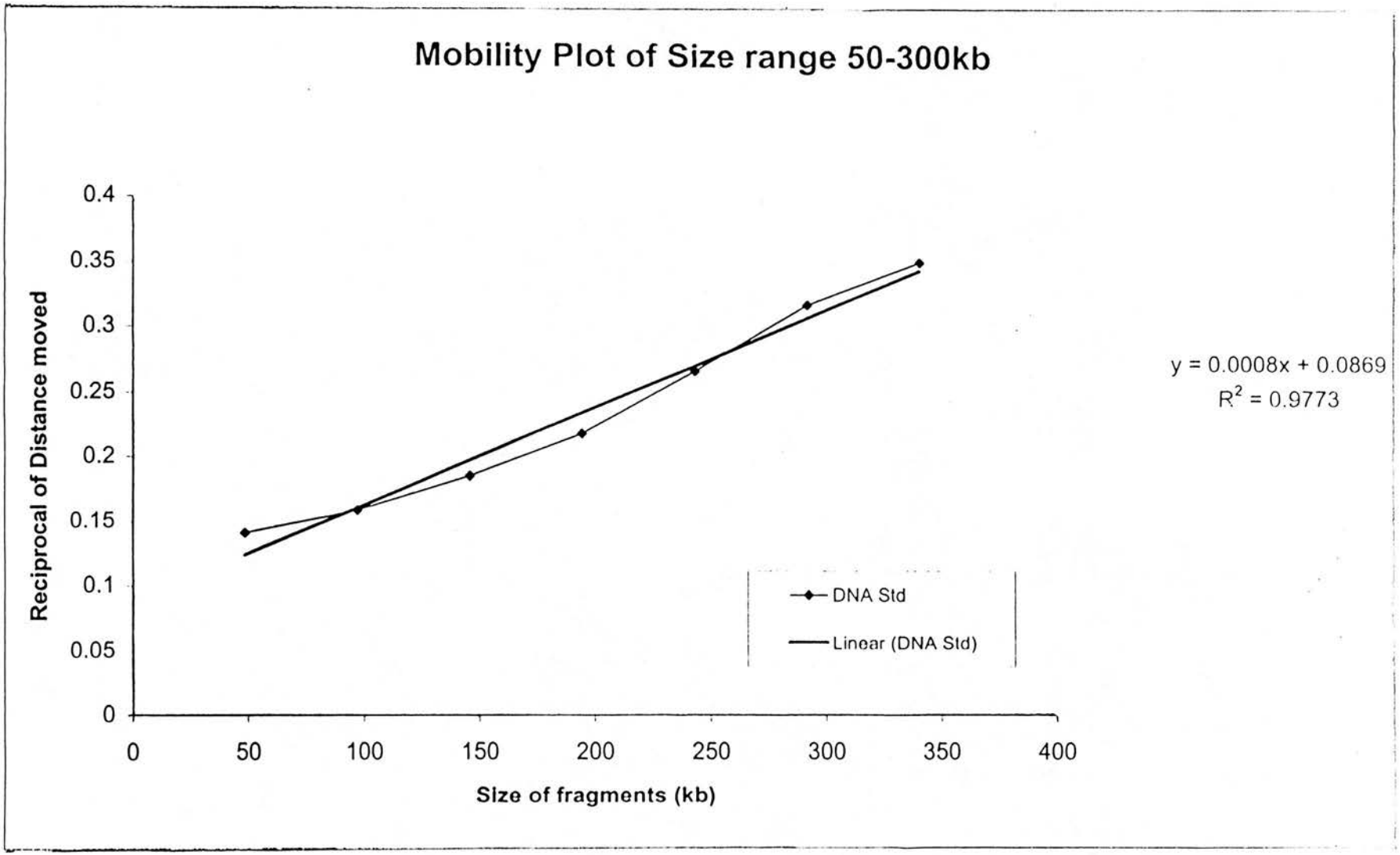
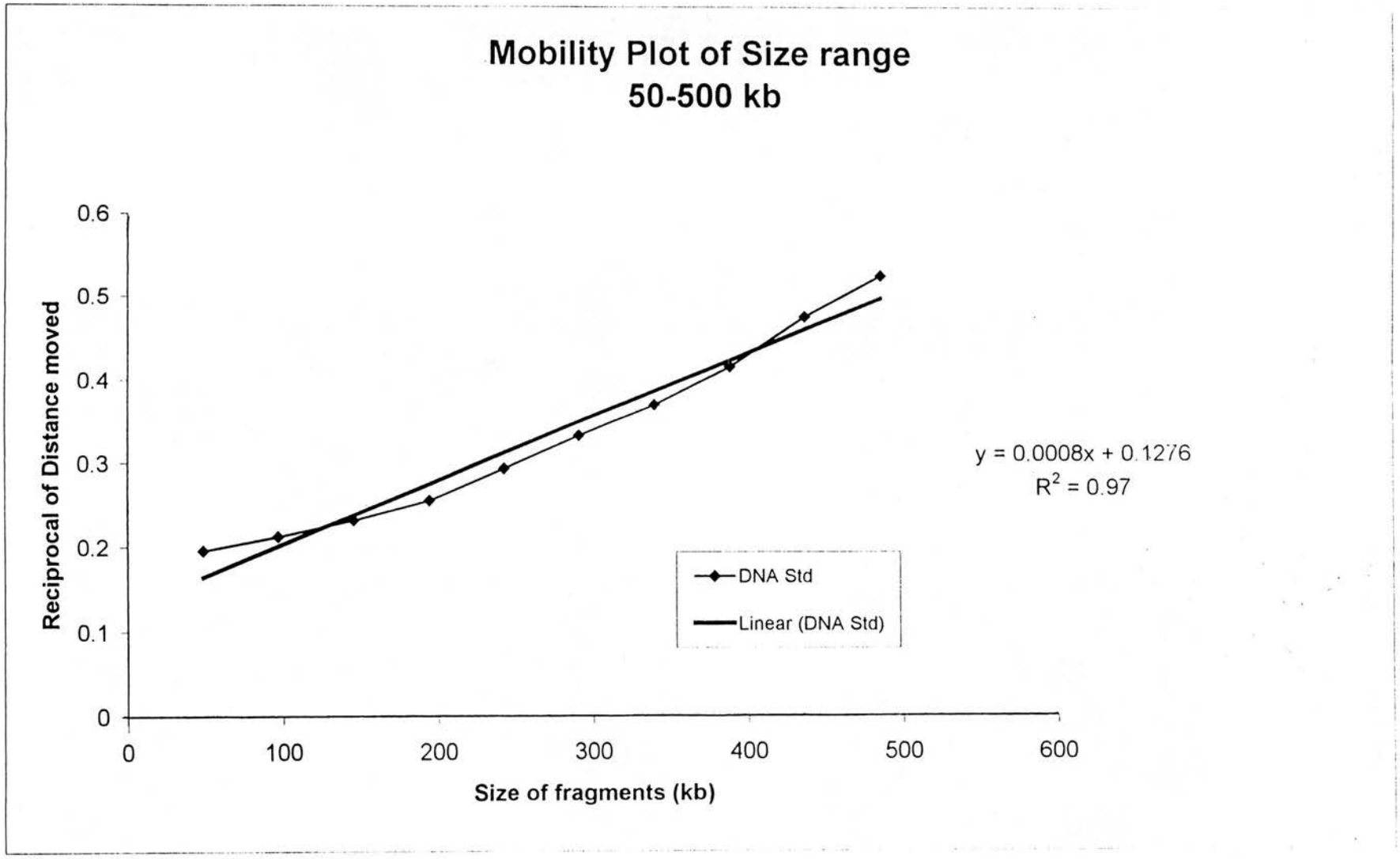
Appendix 1 Exploded view of the Transverse Alternating Field Electrophoresis



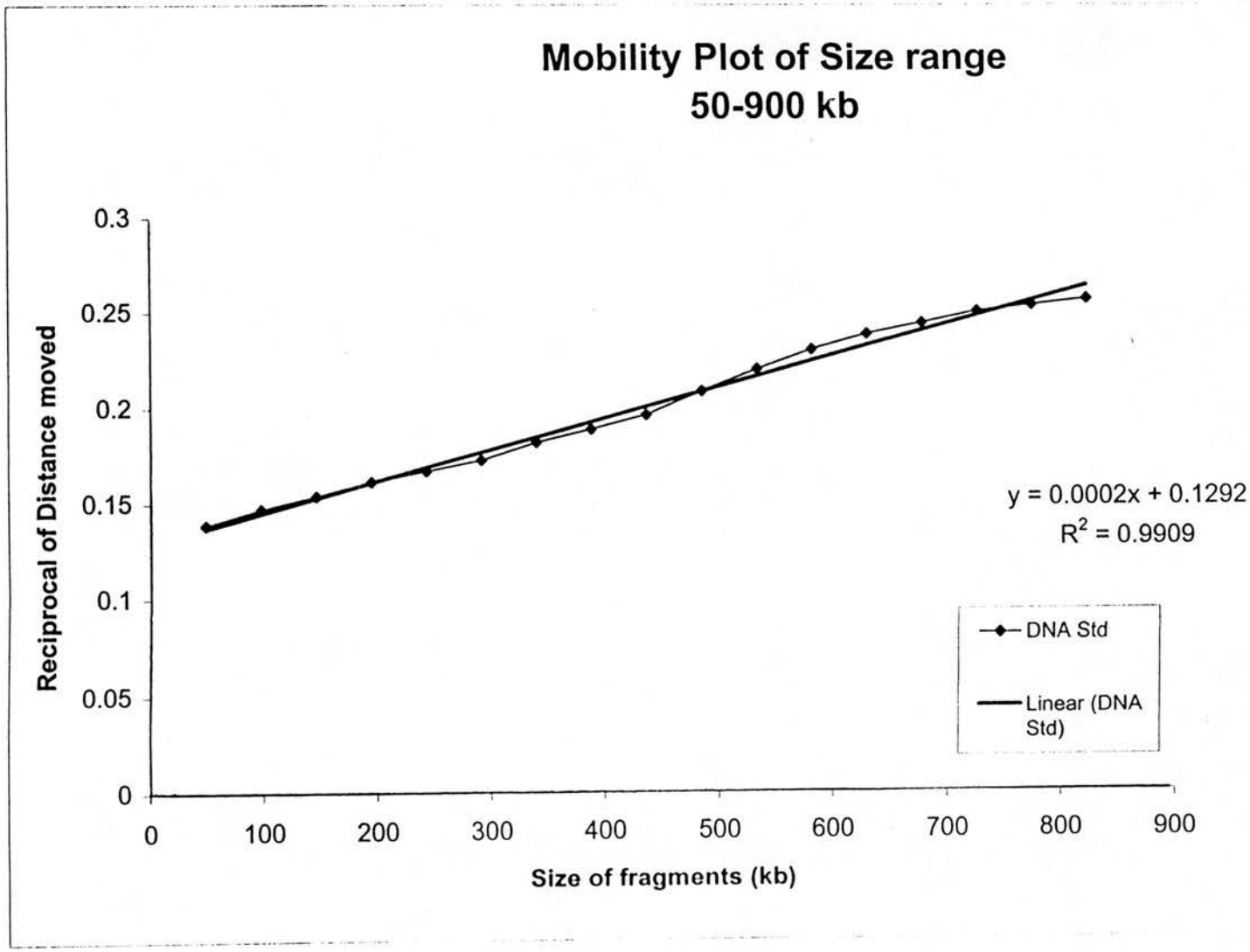
Appendix 2


Mobility Plot of Size range 50-300kb
Reciprocal of Distance moved
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
50
100
150
200
250
300
350
400
Size of fragments (kb)
DNA Std
Linear (DNA Std)
y = 0.0008x + 0.0869
R2 = 0.9773

Appendix 3


Mobility Plot of Size range
50-500 kb
Reciprocal of Distance moved
0.6
0.5
0.4
0.3
0.2
0.1
0
0
100
200
300
400
500
600
Size of fragments (kb)
DNA Std
Linear (DNA Std)
y = 0.0008x + 0.1276
R2 = 0.97

Appendix 4


Mobility Plot of Size range
50-900 kb
Reciprocal of Distance moved
0.3
0.25
0.2
0.15
0.1
0.05
0
0
100
200
300
400
500
600
700
800
900
Size of fragments (kb)
y = 0.0002x + 0.1292
R2 = 0.9909
DNA Std
Linear (DNA Std)